The ecology and growth of the netted dogwhelk Nassarius reticulatus (Gastropoda: Nassariidae)

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The ecology and growth of the netted dogwhelk *Nassarius reticulatus* (Gastropoda: Nassariidae)

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Abstract

The distribution of *Nassarius reticulatus* around Anglesey (North Wales, UK) revealed well established populations along the west coast, smaller populations along the north coast and a complete absence of this gastropod from the east and south coast. *Nassarius reticulatus* is well adapted to varying degrees of wave exposure and prefers fine sand sediments, with <0.5% organic and <2% silt content. New recruits appear in the population at a size of 7-8.9 mm between February and April one year after hatching. Mortality was higher in an intertidal lagoon than in a subtidal beach in Rhosneigr, especially during summer. Whelks from the intertidal lagoon had a higher L_∞ and a lower K value than whelks from the subtidal beach in Rhosneigr. *Nassarius reticulatus* forms taller and thicker shells when inhabiting sheltered areas, which are more resistant to predation pressure. Growth rate increased with increasing seawater temperature. Smaller individuals allocated more energy to growth, while larger whelks invested more energy in reproduction. Shell growth rate was similar between males, females and imposex-affected females. Limitations in food availability suppressed shell growth and reproductive output. Length frequency analysis overestimated the shell growth of *N. reticulatus*, due to overlapping older size-classes. Estimation of the age and growth of *N. reticulatus* from statolith growth rings was a more reliable method than length frequency analysis. The first prominent statolith ring is formed during larval settlement and subsequent rings are formed annually. Spawning of *N. reticulatus* in the laboratory occurred throughout the year, with a peak during July. Breeding size classes of *N. reticulatus* have a shell length $\geq$ 21 mm. *Nassarius reticulatus* produced smaller egg capsules containing smaller eggs, which had a shortened embryonic development and produced smaller larvae during the summer. Hatching occurred successfully over a range of seawater temperatures (8.5-20°C), however, larvae only metamorphosed at temperatures >16.3°C. Lack of a suitable substrate for settlement reduced the growth rate of the larvae and delayed metamorphosis. Imposex examination in sympatric populations of *N. reticulatus* and *Nucella lapillus* indicated that both gastropod species were highly affected by TBT pollution in areas adjacent to commercial ports and small marinas.

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CHAPTER 1

General Introduction

Molluscs arguably constitute the largest and most diverse invertebrate phylum aside from the arthropods. Over 50,000 living and 35,000 fossil species have been described as members of the phylum Mollusca (Ruppert & Barnes, 1996). The long geological history and the well preserved mineral shells of molluscan species have resulted in a rich fossil record that dates back to the Cambrian (Ruppert & Barnes, 1996). Molluscs are unsegmented, bilaterally symmetrical animals, and despite the striking differences amongst clams, snails and cephalopods, all molluscs are built on the same fundamental plan (Ruppert & Barnes, 1996). The phylum Mollusca is divided into seven classes that include living species, three of which are regarded as major classes (i.e. the Gastropoda, Bivalvia and Cephalopoda). Considering the wide variety of habitats that members of the class Gastropoda have invaded, they are the most successful of the molluscan classes. Gastropods occur in terrestrial, freshwater, and marine ecosystems, and in the latter case species have become adapted to every kind of benthic and pelagic habitat (Hayward & Ryland, 2000). The evolution of gastropods involved four major adaptations: a) development of the head, b) dorsoventral elongation of the body, c) formation of a stronger and more protective shell, and d) torsion during larval development (Ruppert & Barnes, 1996). The Gastropoda are classified into three subclasses: the Prosobranchia, the Opisthobranchia, and the Pulmonata (Ruppert & Barnes, 1996; Hayward & Ryland, 2000).

Nassarius (Hinia) reticulatus (Linnaeus, 1758) is a member of the family Nassariidae (Prosobranchia: Neogastropoda) that derived from either the late Cretaceous or early Tertiary period (Taylor *et al.*, 1980). The opaque coiled shell of *N. reticulatus* is oval-conical, up to 30mm height (Hayward & Ryland, 2000) and about 14 mm in width (McMillan, 1968; Fretter & Graham, 1984). The shell is covered with a distinct light brown periostracum with a dark band on the subsutural part of each whorl (Fretter & Graham, 1984). The whorls are almost flat-sided and have closely spaced ribs cut by fine spiral grooves that give a square, block like, reticulate pattern to the shell (Hayward & Ryland, 2000). The outer shell lip is thin and wrinkled, but in mature animals it thickens internally and forms a ridge with 6-9 teeth (Fretter & Graham, 1984). The colour of the animal is brownish with black and white spots, and the head is flattened and bears a pair of cephalic tentacles and eyes, which appear to detect only changes in general light intensity (Fretter & Graham, 1984; Hayward & Ryland, 2000). Behind and dorsal to the head is the mantle cavity in which the gills (ctenidia), the associated sense organ (osphradium) and the mucus gland (hypobranchial gland) are located (Hayward & Ryland, 2000). The siphon is on the left side of the mantle and projects a long way from the canal in active animals (Fretter & Graham, 1984). The mouth and the radula are at the end of a long eversible proboscis, which has a sucker action (Fretter & Graham, 1994). The foot is rather long and has well-developed antero-lateral horns (Hayward & Ryland, 2000). *Nassarius reticulatus* is a very active and agile animal due to the high level of organisation of its columellar muscle (Fretter & Graham, 1984). It can move its shell on its foot rapidly and crawl very fast, in order to locate carrion or avoid predators (Fretter & Graham, 1984). About $1000 \cdot mm^{-2}$ endings of unicellular chemo- and mechanoreceptors are present on the unspecialised external epithelium of *N. reticulatus,* and these are most abundant on the anterior border of the foot and on the cephalic tentacles (Crisp, 1971). Photoreceptors located on the external epithelium of the siphon are responsible for contraction of the siphon muscles, and others located on the edge of the mantle are responsible for contraction of the columellar muscle (Crisp, 1972). The ability of the siphon to respond to shadows has a clear adaptive value for *N. reticulatus* which spends long periods of inactivity buried in the sand with only the siphon tip exposed, and can reduce predation by blennies and other bottom feeders (Crisp, 1972).

Nassarius reticulatus can be found from Morocco, Madeira, the Canary Islands and Azores to northern Norway, throughout the Mediterranean and the Black Sea (Fretter & Graham, 1984; Hayward & Ryland, 2000). It is a common species on all British coasts but it is rare in central parts of the Irish Sea and on the east coast of Scotland (Fretter & Graham, 1984). It is a euryhaline (9-45‰) and eurythermic (0-33°C) species and is very well adapted to low sublittoral and intertidal areas (up to 15 m depths), which are characterised by strongly varying physical environmental factors (Eriksson & Tallmark, 1974; Eriksson *et al.*, 1975). *N. reticulatus* is common around rocky shores, where they are found on patches of sand and fine gravel (Bouchet *et al.*, 1979; Fretter & Graham, 1984), in *Zostera* beds (Eriksson *et al.*, 1975; Bouchet *et al.*, 1979; Tallmark, 1980; Lambeck, 1982), *Mytilus edulis* banks (Tallmark, 1980) and where freshwater streams run into the sea (Barnett *et al.*, 1980). The whelks prefer well oxygenated areas rather than dense black mud, although they like habitats rich in animal detritus (Fretter & Graham, 1984). *N. reticulatus* is a scavenger feeding on carrion washed in during high tides and on recently dead or moribund organisms in the upper shore (Britton & Morton, 1994). They prefer relatively sheltered habitats, as strong water movements may constrain carrion detection and reproduction (Lambeck, 1982). Scavengers may frequently experience extended starvation periods, since carrion is an ephemeral and unpredictable food source (Britton & Morton, 1994).

Differences in growth rate between gastropod populations from different localities may be attributed to environmental conditions such as seawater temperature, food quantity and quality, intertidal height and population density (Vermeij, 1980; Hughes, 1986). Shell growth rate increases when the temperature is higher during the summer months in several gastropod species (Scheltema, 1964; Lambeck, 1984; Morton & Chan, 2004; Barroso *et al.*, 2005a). Limited food supply retards growth of individuals due to the reduced rate of secretion of shell material during starvation periods (McKillup & Buttler, 1983; Hughes, 1986). Also growth rates decline with age as more energy is allocated to reproduction, and cessation of shell growth at the stage of sexual maturity is commonly accompanied by a pronounced thickening of the apertural margin, often with tooth-like sculpturing of the inner rim (Hughes, 1986). The adaptive significance of shell shape has been related to shore exposure (Seed, 1978; Smith, 1981b), predation (Hughes & Elner, 1979), locomotion (Linsley, 1978) and the type of substratum (Palmer, 1980). Usually gastropods inhabiting wave-exposed shores are characterised by smaller and broader shells, with greater aperture and foot size (Murty & Balaparameswara Rao, 1978; Smith, 1981b; Lam & Calow, 1988) as an adaptation to withstand water currents. Predation pressure is higher along sheltered shores, thus gastropods inhabiting such environments have typically larger and thicker shells with a narrower aperture (Boulding & Van Alstyne, 1993).

The demographic characteristics of a population, such as abundance, age structure, birth rates, age specific growth rates and mortality, reflect the ecology of a species and can provide information about the spatial and temporal extent of environmental stresses (Cerrato, 1980). Population attributes can be determined using graphical or computerised methods for analysing size-frequency distributions (Cerrato, 1980; Lambeck, 1984; Morton & Chan, 2004; Barroso *et al.*, 2005a). Such methods are based on the aspect that each modal class will correspond to a cohort and represent different age classes determined at regular intervals (Morales-Nin, 1992). However, the number of clearly defined cohorts can be reduced by poor recruitment in some years, variation in the duration of the breeding season, mortality, or if sampling takes place at a time long after the most recent spawning event (Cerrato, 1980; Grant *et al.*, 1987). Older age groups usually tend to merge because the variation in size between individuals increases with age, while growth rates decline (Cerrato, 1980; Grant *et al.*, 1987).

Early attempts to determine the age and growth of gastropods relied mainly on morphological characteristics of the shell, such as thickness of the shell lips and the formation of teeth on the inner lip margins (Berry & Crothers, 1968; Feare, 1970a; Coombs, 1973; Wood & Olsen, 1983; Appeldoorn, 1988). However, such methods can under-estimate the age of gastropods due to the expected overlap between adjacent age-classes (Coombs, 1973) or due to erosion of the edge of the lip over time (Appeldoorn, 1988; Stoner & Sandt, 1992). External shell growth marks have also been used to estimate growth and age of gastropod species (Bretos, 1980; Picken, 1980, Tallmark, 1980; Williamson & Kendall, 1981; Barroso *et al.*, 2005a), though such marks become less discernible in older gastropods when shell growth decreases. In addition, the shell margin is frequently damaged and repaired resulting in the production of growth checks, which can be difficult to distinguish from those of annual origin (Richardson *et al.*, 2005b). Based on the number of shell rings Tallmark (1980) suggested that the maximum age attained by *Nassarius reticulatus* in Sweden was at least 15 years, while Bryan *et al.* (1993) reported a maximum of 17 annual checks from a population in SW England. The formation of internal shell micro-growth bands has also been used for age determinations in several gastropod species (Ekaratne & Crisp, 1982; 1984; Prince *et al.*, 1988a; Erasmus *et al.*, 1994; Shepherd *et al.*, 1995a; 1995b; Barroso *et al.*, 2005a). However, the method has not been widely applied to studies of mesogastropod and neogastropod growth, since the conical spired shell of such species is not suitable for producing a continuous growth sequence without repeated serial sectioning (Richardson, 2001).

A new development in the study of growth and ageing of gastropod species is the identification of periodic growth increments in statoliths, which are structures similar to fish otoliths. Gastropod statoliths are found in the statocysts, the sense organs for equilibrium, which provide the animal with information on the direction of gravity with reference to the animal's position (Dorsett, 1986). The statocysts contain statolymph, in which calcareous particles are floating, either one statolith (neogastropods, marine mesogastropods) or several small statoconia (archaeogastropods, freshwater mesogastropods) (Fretter & Graham, 1994). Statoliths have been extensively used, especially during the last 20 years, as tools for ageing several cephalopod species (Jackson, 1994). Arkhipkin & Bizikov (1997) proposed that since increments are deposited periodically in statoliths of most squid species, they can give information regarding their age, growth rate and ontogenetic events during the life cycle of the animal. Confirmation of the daily periodicity of statolith increment formation in squids has been achieved by placing a chemical mark in the statolith and then comparing the number of days elapsed with the number of increments produced after marking (Rodhouse & Hatfield, 1990; Jackson, 1994). Until recently only a few studies had described the use of growth rings in gastropod statoliths as a tool for determining the age of the larvae (Bell, 1982; 1983; 1984; Grana-Raffucci & Appeldoorn, 1997; Zacherl *et al.*, 2003) and adults (Karageorgopoulos, 1997; Saurel, 2002; Barroso *et al.*, 2005b; Richardson *et al.*, 2005a; 2005b). Attempts have been made to explain the periodicity of ring formation in the statoliths of gastropod species during these early studies but no strong experimental evidence has yet been provided. The first ring formed on the statolith has been related either to hatching (Karageorgopoulos, 1997; Saurel, 2002; Zacherl *et al.*, 2003) or settlement (Richardson *et al.*, 2005a; Barroso *et al.*, 2005b) of gastropod larvae, and there are strong indications that subsequent prominent rings are deposited annually.

Nassarius reticulatus is sexually mature at the age of ~ 4 years old (Tallmark, 1980; Bryan *et al.*, 1993). The sexes are separate and reproduction involves copulation and internal fertilisation (Hayward & Ryland, 2000). The breeding period of *N. reticulatus* varies throughout Europe, but usually takes place in inshore waters during spring and summer (March-August), and more rarely during autumn and winter (Lebour, 1931; Fretter & Graham, 1984). Differences regarding the period of egg capsule deposition between geographically separated populations of *N. obsoletus*, have been related to the time when the required environmental temperature to initiate spawning was reached in the various regions (Sastry, 1971; Tallmark, 1980). Reproductive effort and fecundity of gastropods can be affected by food availability (McKillup & Butler, 1979; Cheung & Lam, 1999), salinity (Cheung, 1997) and parental size (Chia & Skeel, 1973; Spight & Emlen, 1976; Chester, 1996). The eggs of *N. reticulatus* are packed inside small, flattened capsules, which are often laid on *Zostera*, red algae, shells and stones (Lebour, 1931; Barnett *et al.*, 1980; Fretter & Graham, 1984;). The egg capsules of *N. reticulatus* are 4.5-5 mm in height and about 4 mm across and they contain ~50-350 eggs, 160 um in diameter, each in its own covering but all floating in a common nourishing fluid contained in the capsule (Fretter & Graham, 1984). The embryos of *N. reticulatus* hatch 1-2 months after deposition of the egg capsules to release free swimming planktotrophic larvae (Lebour, 1931; Fretter & Graham, 1984).

The period of pelagic larval development of marine invertebrates is influenced by environmental factors such as food supply, temperature and salinity (Davis, 2000). Usually the planktonic phase of *Nassarius reticulatus* from hatching to settlement requires 2-3 months (Lebour, 1931; Fretter & Graham, 1984). The newly hatched *N. reticulatus* veligers have a well formed transparent shell consisting of one whorl, and a bilobed velum with a pigmented border (Lebour, 1931). The veligers feed continuously, by rising through the water with their velum fully extended and collecting food particles, then withdrawing the velum and sinking in order to rise again (Fretter, 1984). After a few weeks the velum becomes 4-lobed (Lebour, 1931; Fretter & Graham, 1984), and later, when the foot is well developed, the larvae are able to swim or crawl at will. When the larvae are close to metamorphosis they spend more time near to the seabed exploring the sediment (Crisp, 1974; Hughes, 1986). Metamorphosis involves a sequence of fast synchronised non-reversible alterations and is determined by the loss of the velum (Crisp, 1974). Metamorphosis occurs either as an increased sensitivity to some external inducing substances present in the seawater, the programmed eventual release of some endogenous stimulatory internal factor in the absence of external cues, or the degradation of some internal factor inhibiting metamorphosis (Pechenik *et al.*, 1996a). Although larvae may reach the stage where they are competent for metamorphosis they may delay their settlement and continue to grow until a suitable substratum is encountered (Scheltema, 1961; Fretter & Graham, 1994). A favourable substratum is a combination of physical, chemical and biological properties of the sediment (Scheltema, 1961; Fretter & Graham, 1994). Many larvae will colonise new favourable habitats, however, some of them will never encounter a suitable place for settlement (Hughes, 1986).

Antifouling paints have been used to reduce or prevent the settlement of fouling organisms on ship's hulls since the 1960's and the most effective ones are based on triorganotin compounds like tributyltin (TBT) and to a lesser extent triphenyltin (TPT) (Bennett, 1996). In the early 1990's many of the *Nucella lapillus* populations around southern England, France and Norway had declined or were no longer present due to TBT pollution (Stroben *et al.*, 1992b). Binding of TBT on cellular proteins can disturb steroid metabolism (Bettin *et al.*, 1996; Alzieu, 2000), and the increased testosterone levels in females can result in the superimposition of male sex characters (imposex) onto female prosobranchs that may cause sterility in some species (Smith, 1971). The phenomenon of imposex was first described for the dogwhelk *N. lapillus* by Blaber (1970) and since then TBT pollution has been reported to cause imposex in more than 118 prosobranch species (Fioroni *et al.*, 1991). The sediment-dwelling *Nassarius reticulatus* was initially proposed as a bioindicator for TBT pollution by Stroben *et al.* (1992b) and since then many investigators have validated the advantages of using this key-species for monitoring the biological effects of organotin compounds on marine organisms (Bryan *et al.*, 1993; Barroso *et al.*, 2000; 2002). *N. reticulatus* presents a relatively lower sensitivity to TBT pollution than *N. lapillus*, and thus it is a more appropriate species for monitoring heavily contaminated areas (Stroben *et al.*, 1992a; Bryan *et al.*, 1993). Species such as *N. reticulatus*, which have a planktonic veliger stage, exhibit a wide distribution and are also able to recolonise previously polluted areas where the population was extinct or had declined in numbers (Stroben *et al.*, 1992b).

The present study concentrates on a range of several aspects of the biology and ecology of *Nassarius reticulatus*. The first chapter includes a general description of the species and an introduction to the research previously undertaken. Chapter two describes the distribution of *N. reticulatus* around Anglesey (N. Wales) and identifies several environmental factors (sediment type, wave exposure and predation) that appear to promote the establishment of populations. The morphological variability between populations from different habitats and the adaptive significance of different shell phenotypes are investigated. In chapter three the population dynamics (growth, recruitment, mortality) of *N. reticulatus* in an intertidal lagoon and in a subtidal beach at Rhosneigr are described using two different methods of length-frequency analysis. Variations in growth between different seasons, between whelks of different gender, size class and origin, and between whelks maintained under different feeding regimes, are examined under laboratory conditions. Chapter four investigates the formation and periodicity of growth rings in the statoliths of *N. reticulatus* and validates the use of the statolith growth rings for determining the age and the growth patterns of gastropods. In chapter five the spawning behaviour of *N. reticulatus*, the effects of reproduction on shell growth, and the reproductive output of different size classes of whelks maintained under different feeding regimes throughout the year are described. In addition, the effects of temperature on hatching frequency and on the duration of embryonic development are investigated. Chapter six describes the morphology and behaviour of *N. reticulatus* veligers during different developmental stages from hatching to metamorphosis and investigates the effects of temperature on larval development. Factors inducing delayed metamorphosis are also discussed. In chapter seven levels of imposex in *N. reticulatus* populations around Anglesey are investigated and compared with the imposex level in sympatric populations of *Nucella lapillus*. Finally the results of all chapters are discussed together in chapter 8. Cited references from all eight chapters are presented at the back of the thesis.

CHAPTER 2

Distribution and morphometry of *Nassarius reticulatus* around the Isle of Anglesey

2.1 INTRODUCTION

The geographical distribution of *Nassarius reticulatus* extends from Morocco, Madeira, the Canary Islands and Azores to northern Norway, throughout the Mediterranean and the Black Sea (Fretter & Graham, 1984; Hayward & Ryland, 2000). It is a common species on all British coasts but it is rare in central parts of the Irish Sea and on the east coast of Scotland (Fretter & Graham, 1984). *N. reticulatus* is mainly a low sublittoral species but it can also be found from intertidal areas up to 15m depths (Fretter & Graham, 1984). According to Lambeck (1982) the density and biomass of *N. reticulatus* in the saline lake Grevelingen (Netherlands) was higher at 4m depth and gradually decreased into deeper water. *N. reticulatus* is a euryhaline and eurythermic species and is well adapted to the shallow intertidal habitat which is characterised by strongly varying physical environmental factors (Eriksson & Tallmark, 1974). *N. reticulatus* is common around rocky shores on patches of sand and fine gravel, into which they borrow, leaving the tip of the siphon exposed (Bouchet *et al.*, 1979; Fretter & Graham, 1984). *N. reticulatus* also occurs under stones (Fretter & Graham, 1984). Tallmark (1980) recorded a well established population of *N. reticulatus* in Gullmar fjord (Sweden) where the substratum was about 80% fine and very fine sand and in certain parts it was intermingled with gravel and shells. However, Lambeck (1982) indicated that *N. reticulatus* is one of the most widespread macrobenthic animals in lake Grevelingen that shows no distinct preference for any bottom type and can be found in medium coarse sand to silt sediments.

Nassarius reticulatus prefers well oxygenated areas rather than dense black muds, although they like places rich in animal detritus (Fretter & Graham, 1984). Individuals <15 mm are mainly deposit feeders and aggregate in detritus rich substrates, whereas larger snails are mainly scavengers and are attracted to areas with more carrion (Tallmark, 1980). During a two-choice laboratory experiment, 85% of *N. reticulatus* showed a preference for sand substratum against pebble and 75% preferred sand enriched in detritus sediments than clean sand (Eriksson *et al.*, 1975). *N. reticulatus* is very common in *Zostera* beds (Eriksson *et al.*, 1975; Bouchet *et al.*, 1979; Tallmark, 1980; Lambeck, 1982), *Mytilus edulis* banks (Tallmark, 1980) and where freshwater streams run into the sea (Barnett *et al.*, 1980). The abundance of *N. reticulatus* in areas with eelgrass patches and with mussel or cockle banks, which act as traps for detritus, was ~10 times higher than in areas with pure sand (Tallmark, 1980). According to Reise (1982) a well developed population of *N. reticulatus* in the subtidal perennial eelgrass beds in the Wadden Sea disappeared when the eelgrass beds were eliminated after a disease in the 1930s. *N. reticulatus* prefers relatively sheltered habitats richer in animal detritus (Barnett *et al.*, 1980). The elimination of tides in the saline lake Grevelingen, because of the construction of a dam that cut off a connection with the North Sea, allowed colonisation of *N. reticulatus* in the sheltered environment of the lake where an eelgrass vegetation was also developed (Lambeck, 1982). *N. reticulatus* cannot tolerate strong water movements as they find it difficult to detect and reach food, or to reproduce successfully, and this environment usually discourages this gastropod from inhabiting very wave-exposed shores (Lambeck, 1982).

Stenzler & Atema (1977) provide a detailed description of predation on *Nassarius obsoletus* by the green crab *Carcinus maenas* under laboratory conditions. The crab is able to lift the snail with one chela and crush the weak and eroded apex with the other chela. When the apex is finally crushed it is raised to the mandibles and small parts of flesh and shell are torn off. Further shell crushing and feeding continues for a period of ~ 30 minutes after which most of the shell is often completely destroyed (Stenzler & Atema, 1977). According to Gore (1966) when the tube feet of an active potential predator, like the sea star *Luidia alternata*, came into contact with the siphon of *N. vibex* a violent flipping escape reaction occurred. Snails which were buried in a resting position emerged immediately from the sediment and began continual thrusts of the foot, which is stretched out and becomes thin, and then was used as a lever to push the snail over in a series of somersaults (Gore, 1966). Also carnivorous gastropod species, such as members of the families Naticidae and Buccinidae, have been reported to feed on *Nassarius* species after drilling distinctive round holes through the shells. The moon snail *Lunatia heros* covers the shell of *N. obsoletus* with mucus and then bores a hole through the shell and feeds on the flesh with its radula at the end of its proboscis (Stenzler & Atema, 1977). The whole process may take several days to complete (Stenzler & Atema, 1977). Gore (1966) reported that the presence of the Fasciolariidae gastropods *Fasciolaria hunteria* and *F. tulipa* induced a flipping escape reaction in *N. vibex*.

Most gastropods do not conform to the idealised condition of constancy of shape during growth, thus shape changes as the shell grows and a certain degree of allometry is evident (Vermeij, 1980). If an organism remained geometrically similar during all stages of growth, the surface to volume ratio would be decreased and the effectiveness of surface-dependent properties (respiration, locomotion) would be lower (Vermeij, 1978). Allometric changes in shape may be accompanied by changes in colour and shell sculpture. In many cases, spiral or axial ornamentation present on the earlier formed whorls is reduced or entirely lost on later whorls (Vermeij, 1980). Doming is the type of allometry during which the relative spire height increases during ontogeny and this type of allometry can be recognized by the convex lateral profile of the spire (Vermeij, 1980). Rounding of the aperture and doming reduces the rate at which the foot area decreases with foot volume as the snail grows, thus allowing larger snails to cling to substrata almost as effectively as small individuals (Gould, 1968).

Great variability in the shell size of adult gastropods has been observed within and amongst populations of several species that are characterised by the cessation of their shell growth with maturity (Vermeij, 1980). Animals that achieve a greater size faster are able to compete more effectively against other individuals and also become immune from predation or other mortality causes that affect primarily the smaller sizes (Vermeij, 1980). Such differences in growth rate, and as a result in shell size, between populations from different localities may be attributed to environmental conditions such as seawater temperature, food quantity and quality, intertidal height and population density (Vermeij, 1980).

Variation in shell length between conspecifics inhabiting different environments has been observed in several gastropod species, such as *Cabestana spengleri* (Laxton, 1970), *Cerithium nodulosum* (Yamaguchi, 1977), *Potamopyrgus jenkinsi* (Dorgelo & Leonards, 2001) and has been correlated with differences in food availability. The adult size of gastropods in the more productive waters of the eastern Pacific is 20-50% greater than the size of closely related species in the tropical western Atlantic (Vermeij, 1978). In addition, differences in the mean size of the predatory whelk *Morula marginabla* between different shores were attributed to the different prey-species available on each shore (Moran *et al.*, 1984). Individuals of the crown conch, *Melongena corona*, found in oyster beds along the north Florida coast attained a larger shell size compared to individuals found in adjacent salt marshes feeding on mussels (Bowling, 1994).

Variation in growth and shell size of intertidal gastropods has also been observed between animals inhabiting different shore levels and has been correlated with food availability (Takada, 1995), time available for foraging (Vermeij, 1980), predation and physical extremes (temperature, salinity, desiccation) (Vermeij, 1972). For example, mature adults of *Littorina saxatilis* inhabiting the upper shore have a ridged and banded shell and are twice as large as the smooth and unbanded shells of mature individuals inhabiting the lower shore (Johannesson *et al.*, 1997). The larger shell of the upper shore individuals offers protection against predatory crabs, which are more abundant along the upper shore and have a greater preference for small-sized snails (Johannesson *et al.*, 1997).

Growth rate and maximum adult size of gastropods may be lower in areas with higher population density, where intra-specific competition for food and space is increased (Morrisey, 1987). In populations of *Hydrobia ventrosa* characterised by high densities it has been observed that repeated contact with other snails may influence the feeding behaviour of an individual (Levinton, 1979). The growth rate of the mud snail *H. ulvae* was significantly reduced when the density of the snail population was higher than 0.3 snails·cm^{-2} (Morrisey, 1987).

The adaptive significance of shell shape has been related to shore wave-exposure (Seed, 1978; Smith, 1981b), predation (Hughes & Elner, 1979), locomotion (Linsley, 1978) and the type of substratum (Palmer, 1980). Similar shell morphologies of different gastropod species have evolved in similar types of habitats by convergent evolution (Johannesson, 2003). The aperture and foot size of *Littorina saxatilis* was greater in gastropods of wave-exposed shores than conspecifics from sheltered shores (Smith, 1981b). Individuals of *L. rudis*, which usually inhabit sheltered shores, had smaller apertures and foot size than *L. saxatilis* (Smith, 1981b). Similarly, individuals of the estuarine snail *Neritina violacea* from a sheltered environment had broader shells with a relatively narrow aperture, whereas snails from a wave-exposed area had narrow shells with a relatively wider aperture (Murty & Balaparameswara Rao, 1978). The gastropod *Lymnaea pelegra* from lotic habitats (running waters) is characterised by a larger aperture and thus bigger foot as an adaptation to withstand water currents, compared with individuals from lentic habitats (standing waters) (Lam & Calow, 1988).

Boulding & Van Alstyne (1993) reported that two *Littorina* species from wave-exposed shores were typically smaller and thinner-shelled with a larger foot than gastropods from sheltered shores. When individuals of the wave-exposed-shore species were transplanted into a protected shore they suffered increased mortality, because due to their small size, wide aperture and thin shell they were significantly preferred by shore crabs (Boulding & Van Alstyne, 1993). Individuals of the sheltered shore species, which had a narrower aperture, were more susceptible to dislodgment in a flow tank experiment (Boulding & Van Alstyne, 1993). A narrower aperture is produced by a more elongated spiral growth form, which reduces the relative area of the foot (Hughes & Elner, 1979).

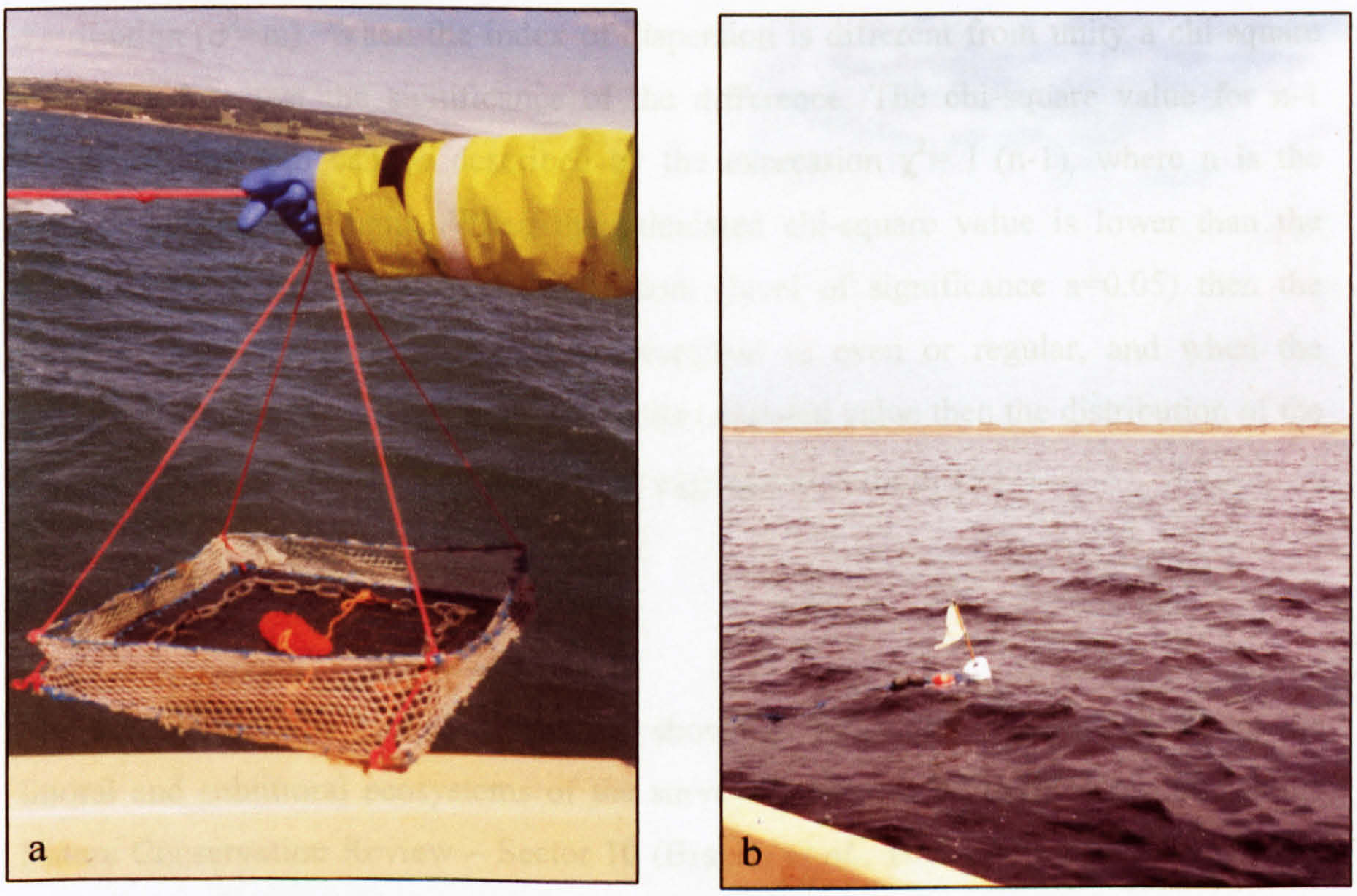
The aims of the present study were: 1) To describe the distribution of *Nassarius reticulatus* around the Isle of Anglesey in North Wales, 2) to identify which factors could inhibit the establishment of a *N. reticulatus* population in certain areas, 3) to determine the optimum benthic conditions for the development of a population, such as depth, mean sediment grain size and organic and silt content in the sediment, 4) to outline the environmental constraints that the population can withstand regarding wave-exposure and predation, 5) to identify the allometric relationships and morphological variability between populations of *N. reticulatus* in different habitats, 6) to correlate morphological variability with environmental conditions such as wave-exposure and predation, and 7) to explain the adaptive significance of different shell phenotypes.

2.2 MATERIALS AND METHODS

2.2.1 Sampling

The distribution and abundance of *Nassarius reticulatus* were investigated at 26 subtidal locations around Anglesey in July 2003. The distribution of *Nassarius* populations appears to be clumped because these opportunistic scavengers are aggregated at locations where carrion sources are detected. As a result the density of *N. reticulatus* per unit area could not be accurately estimated and therefore the evaluation of population abundance in each site was performed with timed samplings. A baited trap was constructed with a metal frame grid (46 x 46 cm) onto which a piece of mackerel was attached in order to attract the animals (figure 2.1a). The bait was wrapped with a piece of plastic net in order to reduce consumption by crabs and a mesh net was fixed around the metal frame in order to avoid losing collected gastropods while pulling up the traps. A metal chain was fixed onto the grid to weight down the trap. Each trap was attached to a long rope with a flagged buoy thus permitting total submersion of the trap on the seabed (figure 2.1b).

Figure 2.1 a) Metal frame trap baited with a piece of mackerel wrapped in plastic net. b) Deployment of traps with flagged buoys in the water.



A transect line of 5-10 traps (depending on the area covered) was submerged in the water for a period of 30 minutes in each site. At some sampling sites where the number of animals collected was low, submersion of traps was repeated until a sufficient number (>50) of gastropods was collected. The number of *Nassarius reticulatus* caught per trap after 30 minutes was recorded. Subsamples of *N. reticulatus* (50-100) collected from each site were stored in plastic bags and transferred to the laboratory for examination of their morphological characteristics, identification of sex and imposex stage and extraction of statoliths. The number of other species such as crabs, starfish and brittle stars that were collected in each trap together with the *Nassarius* was also recorded. One representative sediment sample was collected from each site using a small hand-operated grab and retained for grain size and organic content analysis. Global Positioning System (GPS) co-ordinates of each sampling site were recorded by a Raytheon Raystar 90 GPS Navigator and bathymetric data were recorded using an Incastec Echopilot Classic Plus echo-sounder.

The index of dispersion (I) is the variance to mean ratio (σ^2/m) and can be used to describe the spatial distribution of a population (Elliott, 1977). When the ratio (I) is equal to unity the distribution of the population can be described as random (Elliott, 1977). The Poisson series can be used as a model for the description of a random distribution ($\sigma^2=m$). When the index of dispersion is different from unity a chi-square test is used to test the significance of the difference. The chi-square value for n-1 degrees of freedom can be described by the expression $\chi^2= I\ (n-1)$, where n is the number of sampling units. When the calculated chi-square value is lower than the tabulated value for n-1 degrees of freedom (level of significance a=0.05) then the distribution of the population can be described as even or regular, and when the calculated chi-square value is higher than the tabulated value then the distribution of the population can be described as clumped or aggregated (Elliott, 1977).

2.2.2 Description of sites

The locations of the sampling sites are shown in figure 2.2. Information about the littoral and sublittoral ecosystems of the surveyed sites were provided by the Marine Nature Conservation Review – Sector 10 (Brazier *et al.*, 1999). The wave-exposure of each site was assessed using the Ballantine Biologically-defined Exposure Scale (Ballantine, 1961) and the levels of boat activity were based on personal observations (see table 2.1). The national grid reference of each site was estimated from the GPS co-ordinates using the Great Britain Ordnance Survey sheet 114 – Landranger Series for Anglesey with a scale 1 : 50000 (table 2.1).

Figure 2.2 Location of sampling sites around the coastline of Anglesey (North Wales, UK). Green dots represent the sampling sites where *Nassarius reticulatus* was present and red dots represent the sites where *Nassarius reticulatus* were not caught in the trap. The numbers in the labels represent the average number of *Nassarius reticulatus* caught per trap during a period of 30 minutes (see table 2.2 in results). The blue arrows indicate the direction of the south-west prevailing winds.

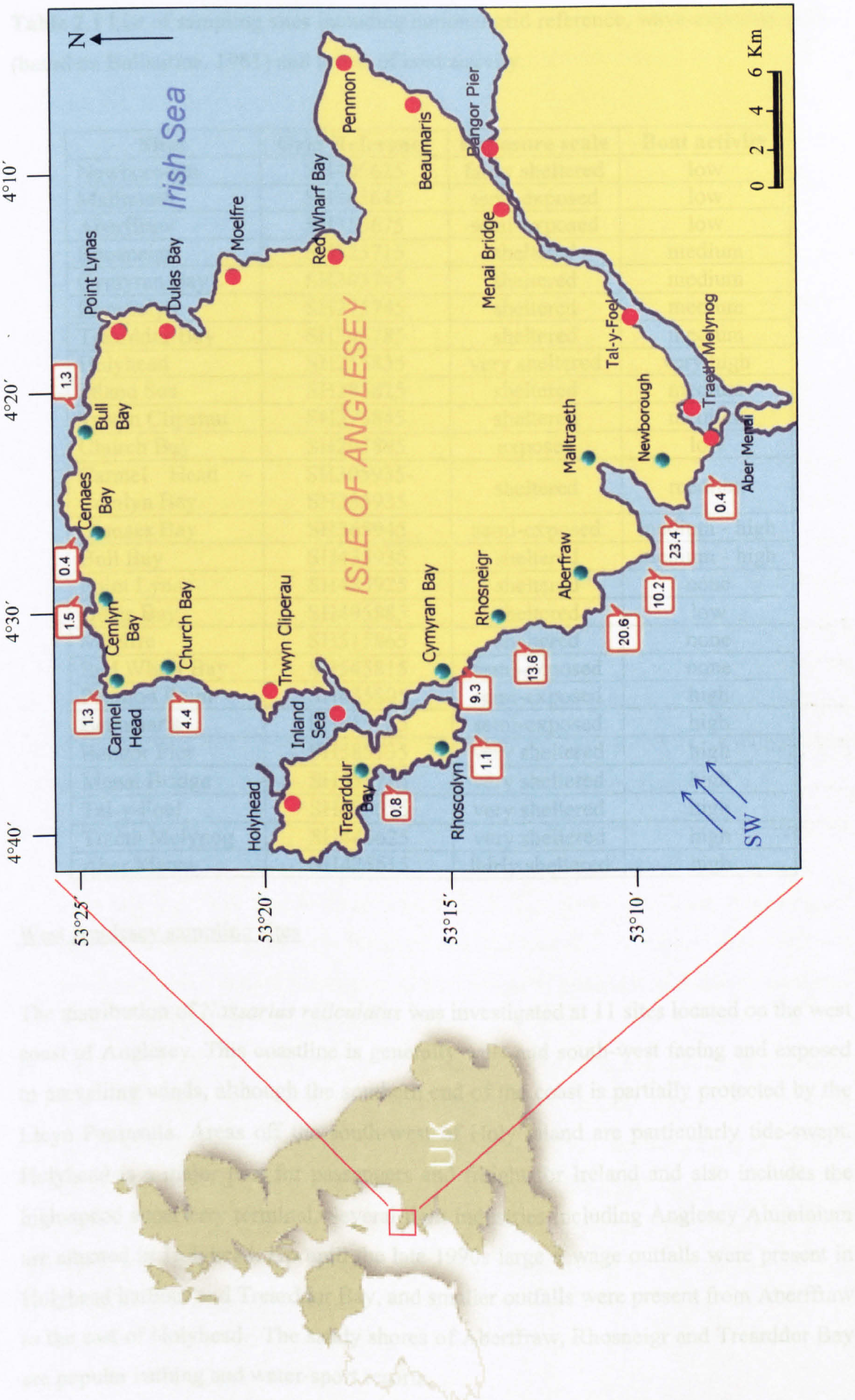

N
Irish Sea
ISLE OF ANGLESEY
Point Lynas
Dulas Bay
Moelfre
Red Wharf Bay
Penmon
Beaumaris
Bangor Pier
Menai Bridge
Tal-y-Foel
Traeth Melynog
Aber Menai
Newborough
Malltraeth
Aberffraw
Rhosneigr
Cymyran Bay
Rhoscolyn
Trearddur Bay
Holyhead
Inland Sea
Trwyn Cliperau
Church Bay
Carmel Head
Cemlyn Bay
Cemaes Bay
Bull Bay
1.3
0.4
1.5
1.3
4.4
0.8
1.1
9.3
13.6
20.6
10.2
23.4
0.4
SW
0 2 4 6 Km
4°10´
4°20´
4°30´
4°40´
53°25´
53°20´
53°15´
53°10´
UK

Table 2.1 List of sampling sites including national grid reference, wave-exposure scale (based on Ballantine, 1961) and levels of boat activity.

Sites	Grid Reference	Exposure scale	Boat activity
Newborough	SH405625	fairly sheltered	low
Malltraeth	SH365645	semi-exposed	low
Aberffraw	SH355675	semi-exposed	low
Rhosneigr	SH325715	sheltered	medium
Cymyran Bay	SH305745	sheltered	medium
Rhoscolyn	SH275745	sheltered	medium
Trearddur Bay	SH245785	sheltered	medium
Holyhead	SH255835	very sheltered	very high
Inland Sea	SH285825	sheltered	medium
Trwyn Cliperau	SH275845	sheltered	medium
Church Bay	SH295895	exposed	low
Carmel Head – Cemlyn Bay	SH305935-SH335935	sheltered	medium
Cemaes Bay	SH365945	semi-exposed	medium - high
Bull Bay	SH435935	sheltered	medium - high
Point Lynas	SH485925	sheltered	none
Dulas Bay	SH495885	sheltered	low
Moelfre	SH515865	sheltered	none
Red Wharf Bay	SH545815	semi-exposed	none
Penmon Point	SH635805	semi-exposed	high
Beaumaris	SH615765	semi-exposed	high
Bangor Pier	SH585735	very sheltered	high
Menai Bridge	SH555715	very sheltered	high
Tal-y-Foel	SH485655	very sheltered	high
Traeth Melynog	SH445625	very sheltered	high
Aber Menai	SH425615	fairly sheltered	high

West Anglesey sampling sites

The distribution of *Nassarius reticulatus* was investigated at 11 sites located on the west coast of Anglesey. This coastline is generally west and south-west facing and exposed to prevailing winds, although the southern end of the coast is partially protected by the Lleyn Peninsula. Areas off the south-west of Holy Island are particularly tide-swept. Holyhead is a major port for passengers and freight for Ireland and also includes the high-speed superferry terminal. Several light industries including Anglesey Aluminium are situated in Holyhead. Up until the late 1990s large sewage outfalls were present in Holyhead harbour and Trearddur Bay, and smaller outfalls were present from Aberffraw to the east of Holyhead. The sandy shores of Aberffraw, Rhosneigr and Trearddur Bay are popular bathing and water-sport resorts.

1. Newborough: The shore consists of mobile sand or sublittoral fine sand with polychaetes and amphipods where sand-scoured rock with kelps and scour-tolerant algae are present.
2. Malltraeth: The shore consists of mobile sand with amphipods and polychaetes and muddy sand with echinoderms. Malltraeth Bay is at the entrance to the Cefni estuary and Malltraeth Sands.
3. Aberffraw: The biotopes of the sandy beach at Aberffraw are typical of moderate wave-exposed sites supporting only a sparse infauna. The surrounding shore consists of littoral rock with barnacles, fucoids, red algae, kelps and scour-tolerant algae. The sublittoral area is characterised by tide-swept silty rock with ascidians and muddy sand areas with echinoderms.
4. Rhosneigr: Many species occur in rockpools, crevices, overhangs and boulders. In general the sand-scoured biotopes such as Newborough and Malltraeth support fewer species than rocky shores. The Rhosneigr shore consists of littoral rock with dense fucoid algae, kelps and scour-tolerant algae or mobile sand with amphipods and polychaetes. The sublittoral area consists of tide-swept silty rock with ascidians and extensive areas of gravel and sand with bivalves.
5. Cymyran Bay: The area is predominantly sandy with crustaceans and polychaete biotopes. Cymyran shore is characterised by mobile fine sand with amphipods and polychaetes, with a few areas of tide-swept silty rock with ascidians.
6. Rhoscolyn: The shore consists of littoral rock with barnacles, fucoids, red algae and kelp forests and by sublittoral fine sand with polychaetes and amphipods.
7. Trearddur Bay: The site is characterised by littoral rock with dense fucoid algae, sublitttoral muddy sand with bivalves and tide-swept silty rocks with ascidians. Small patches of exposed littoral rock with mussels, barnacles and *Alaria esculenta* are also present along the sides of the bay.
8. Holyhead: Holyhead harbour is wave protected by Holy Island and the breakwater. The area inside the harbour is characterised by muddy sand sediment with polychaetes and bivalves, while the western area outside the breakwater consists of infralittoral rock with kelp forests and tide-swept rock with ascidians.
9. Inland Sea: The Inland Sea is a narrow tidal stretch of water which separates Holy Island from the mainland of Anglesey and is characterised by sublittoral fine sand with polychaetes and amphipods.

10. Trwyn Cliperau: The headland Trwyn Cliperau consists of littoral rock with dense fucoid algae and kelps, together with small patches of mobile sand.
11. Church Bay: Littoral rock with barnacles, fucoids, red algae, kelp forests and small patches of exposed littoral rock with mussels, barnacles and *Alaria esculenta*. The sublittoral area is characterised by tide-swept silty rock with ascidians.

North Anglesey sampling sites

The distribution of *Nassarius reticulatus* was investigated at 4 sites located on the north coast of Anglesey. The north Anglesey shores are sheltered low energy areas with less rich species communities compared with the wave-exposed and tide-swept west shores. However, no detailed information was available about the littoral and sublittoral biotopes along the north coastline of Anglesey. The Wylfa Nuclear Power Station is located on the Wylfa headland, which separates the eastern side of Cemlyn Bay from Cemaes Bay.

12. Carmel Head: A dramatic cliff scenery characterises the area around which the wildest section of coast can be found.
13. Cemlyn Bay: The area is characterised by a long pebbly beach and a lagoon where a nature reserve ideal for bird-watching is located.
14. Cemaes Bay: The surrounding coastline includes a sandy beach with rock pools and lagoons. Maritime activity is centred at the old stone pier where a small port for fishing and leisure boats is located.
15. Bull Bay: The area is characterised by a rocky coastline with a small port and is popular for shore fishing. The chemical plant Great Lakes Chemical Corporation (formerly known as Octel Works) was located in the near town of Amlwch, but has recently closed down (2004).

East Anglesey

The distribution of *Nassarius reticulatus* was investigated at 4 sites located on the east coast of Anglesey. Although the east-facing coast of Anglesey is protected from the prevailing winds, moderately strong tidal streams influence the composition of communities where filter-feeding organisms such as sponges, anthozoans and hydroids thrive. Large amounts of silt in suspension and planktonic blooms increase turbidity all year round. Small ports are present at Moelfre and Benllech.

16. Point Lynas: A few boulders with fucoid algae and a rich under-boulder fauna can be found close to the Point Lynas shore. The sublittoral area is characterised by rocky ecosystems affected by scour and disturbance.
17. Dulas Bay: Dulas Bay is a sheltered estuarine sandflat where species richness and abundance is fairly low. The area is probably affected by high levels of heavy metals in the run off from a disused copper mine. The shore consists of mobile or muddy sand with amphipods, polychaetes, bivalves, brittlestars and hydroids.
18. Moelfre: The shore is characterised by littoral rock with dense fucoid algae, kelps and scour-tolerant algae. The sublittoral area is characterised by rocky ecosystems affected by scour and disturbance.
19. Red Wharf Bay: The area is an extensive estuarine muddy sand shore with polychaetes and bivalves and patches of mobile sand in the middle of the bay.

Menai Strait

The distribution of *Nassarius reticulatus* was investigated at 7 sites located along the Menai Strait. The Menai Strait is a narrow channel approximately 20 km long that separates the north Wales mainland from the Isle of Anglesey. Although sheltered from wave action many areas in the Strait experience very powerful tidal streams reaching over 8 knots during spring tides. High turbidity and high nutrient loading from land runoff support a high biomass of a unique mixture of species dominated by filter-feeding animals. The serious sewage pollution in the waters of the Menai Strait has effectively decreased after the transfer of effluent to a central treatment plant in 1997. A few industrial developments are present in the area such as a brick and plastic works near Caernarfon and a small freight port in Bangor. Several water-based leisure activities such as sailing, water-skiing, jet-skiing, scuba diving and angling are popular in the Menai Strait. There are harbours and moorings at Caernarfon, Bangor, Beaumaris and Port Dinorwic. Several species of shellfish such as oysters, clams and mussels are cultivated and harvested from the Menai Strait.

20. Penmon: Penmon headland (Trwyn Du) is composed of limestone and is affected by a moderate amount of wave exposure. The shore consists of littoral rock with dense fucoid algae and in place boulders on sand with sparse algae, littorinids and sponges. The sublittoral ecosystem consists of fine sand with amphipods and polychaetes.
21. Beaumaris: The shore consists of littoral rock with dense fucoid algae and a muddy sand shores with polychaetes and bivalves or fine sand with amphipods and polychaetes.
22. Bangor Pier: At Bangor pier the channel to the Menai Strait narrows and wave action is reduced but due to the increased tidal flow the shores are affected by scour and disturbance. The shore consists of littoral rock with dense fucoid algae, kelps and scour-tolerant algae and in places boulders on sand with sparse algae and littorinids. Mussel beds are present to the west side of the pier. The sublittoral ecosystem consists of fine sand with amphipods and polychaetes.
23. Menai Bridge: The shores near the Menai Suspension Bridge are strongly affected by an increased tidal flow, but wave action is absent. The shore is characterised by muddy sand, coarse gravel and littoral tide-swept rocks with dense fucoid algae, kelps, *Turbularia* species, sponges and anthozoans. The high nutrient loading encourages dense populations of scavenging crustaceans such as crabs and lobsters.
24. Tal-y-Foel: Extensive mussel beds are present on both sides of the Menai Strait at the Sea Zoo site at Tal-y-Foel, together with kelps and scour-tolerant algae. The sublittoral ecosystem consists of fine sand with amphipods and polychaetes.
25. Traeth Melynog: Traeth Melynog is characterised by extensive muddy sand and mobile sand shores with bivalves, polychaetes and amphipods, where a few boulders with scour-tolerant algae, kelps and littorinids are also present.
26. Aber Menai: The shore consists of mobile sand or sublittoral fine sand with polychaetes and amphipods where boulders with sparse algae and littorinids or gravel with mussel beds are present.

2.2.3 Wind direction

Information about wind direction (figure 2.3) along the coast of Anglesey was based on data from the Meteorological Office. The prevailing wind blows between south and west for 56% of the time. Winds only blow from the south-east for ~ 5% of the time.

Figure 2.3 Wind direction on Anglesey as % occurrence at 0900 GTM. Data provided by the Meteorological Office Valley 1981-90. (Source: www.llansadwrn-wx.co.uk/gwynt/windsats.html)

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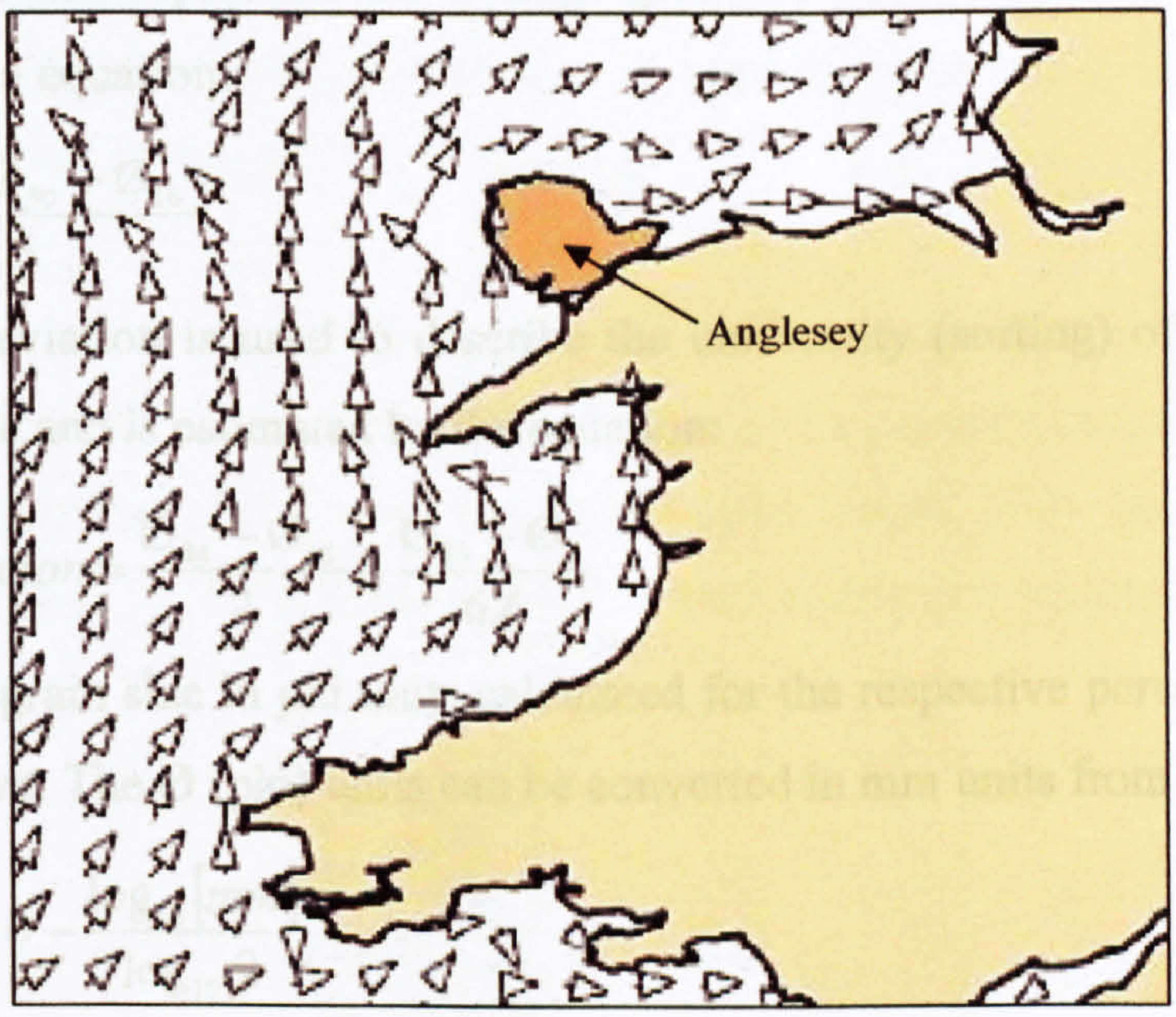
2.2.4 Description of water currents

There is a variable but persistent south-north flow from the coast of Brittany across the mouth of the Irish Sea channel (Slinn, 1974; Pingree & Le Cann, 1989) that also affects the seawater circulation around Anglesey (figure 2.4). Surface and bottom currents carry water fom the southern Irish Sea past the tip of the Lleyn Peninsula and up towards west Anglesey. As the currents reach Anglesey they move along the coast in a north-eastern direction. The net long-term transport through the Irish Sea occurs at an overall speed typically of about 1-2 cm·s^{-1}, although the day-to-day pattern is much more complex (Pingree & Le Cann, 1989). The north-east direction of the residual flow around the coast of Anglesey is affected by wind, tidal and density-driven currents (Horsburgh & Hill, 2003).

2.2.5 Sediment analysis

The collected sediment samples were transferred in labelled plastic bags to the laboratory and immediately stored in a freezer. Prior to sediment grain-size analysis each sample was placed in an evaporating basin and thoroughly defrosted under heating lamps for 2 hours. In order to remove moisture from the samples the evaporating basins were left overnight in an oven at a temperature of 100°C. The next morning

Figure 2.4 Spatial distribution and direction of the mean residual currents around the coast of Anglesey (from Jones & Davies, 2003).



the evaporating basins were left to cool down in a desiccator cupboard for 1 hour. Eighty to 100 g of sediment was placed in a mortar bowl and the grains were gently separated with a pestle. The exact initial dry weight of each sample was measured with a digital scale to the nearest 0.01g. The sediment sample was placed in the top sieve of a stack of brass sieves with decreasing mesh size and the stack was mechanical shaken for 10 minutes. The stack consisted of 11 brass sieves with mesh sizes 2mm, 1.4mm, 1mm, 710um, 500um, 355um, 250um, 180um, 125um, 90um, 63um and a receiver. The cumulative weight of sediment retained within each sieve and the receiver of the stack was measured to the nearest 0.01g. The sample retained in each sieve represents the amount of sediment that was coarser than the respective mesh size, and the sample retained in the receiver represents the amount of sediment that was thinner than the last sieve mesh size (63um).

A cumulative curve with a probability ordinate was constructed by plotting the percentage of cumulative weight of sediment retained in each sieve against the mesh size of that sieve (in mm and phi units) on probability plot paper. The cumulative curve allowed estimation of parameters such as the median, mean and standard deviation of the grain size that can be used to categorise each sediment sample (Folk, 1968). The median grain size represents the grain size of 50% of the grains within a sediment sample and is estimated by the equation: $Median = \varnothing_{50}$

The mean grain size represents the average grain size of a sediment sample and it is estimated by the equation:

$$Mean = \frac{\varnothing_{84} + \varnothing_{50} + \varnothing_{16}}{3}$$

The standard deviation is used to describe the uniformity (sorting) of grain sizes in a sediment sample and is estimated by the equation:

$$Standard\ deviation = \frac{\varnothing_{84} - \varnothing_{16}}{2} + \frac{\varnothing_{95} - \varnothing_{5}}{6.6}$$

where $\varnothing$ is the grain size in phi units calculated for the respective percentages from the cumulative curve. The $\varnothing$ (phi) units can be converted in mm units from the relationship:

$$\varnothing = -\log_2[mm] = -\frac{\log_{10}[mm]}{\log_{10} 2}$$

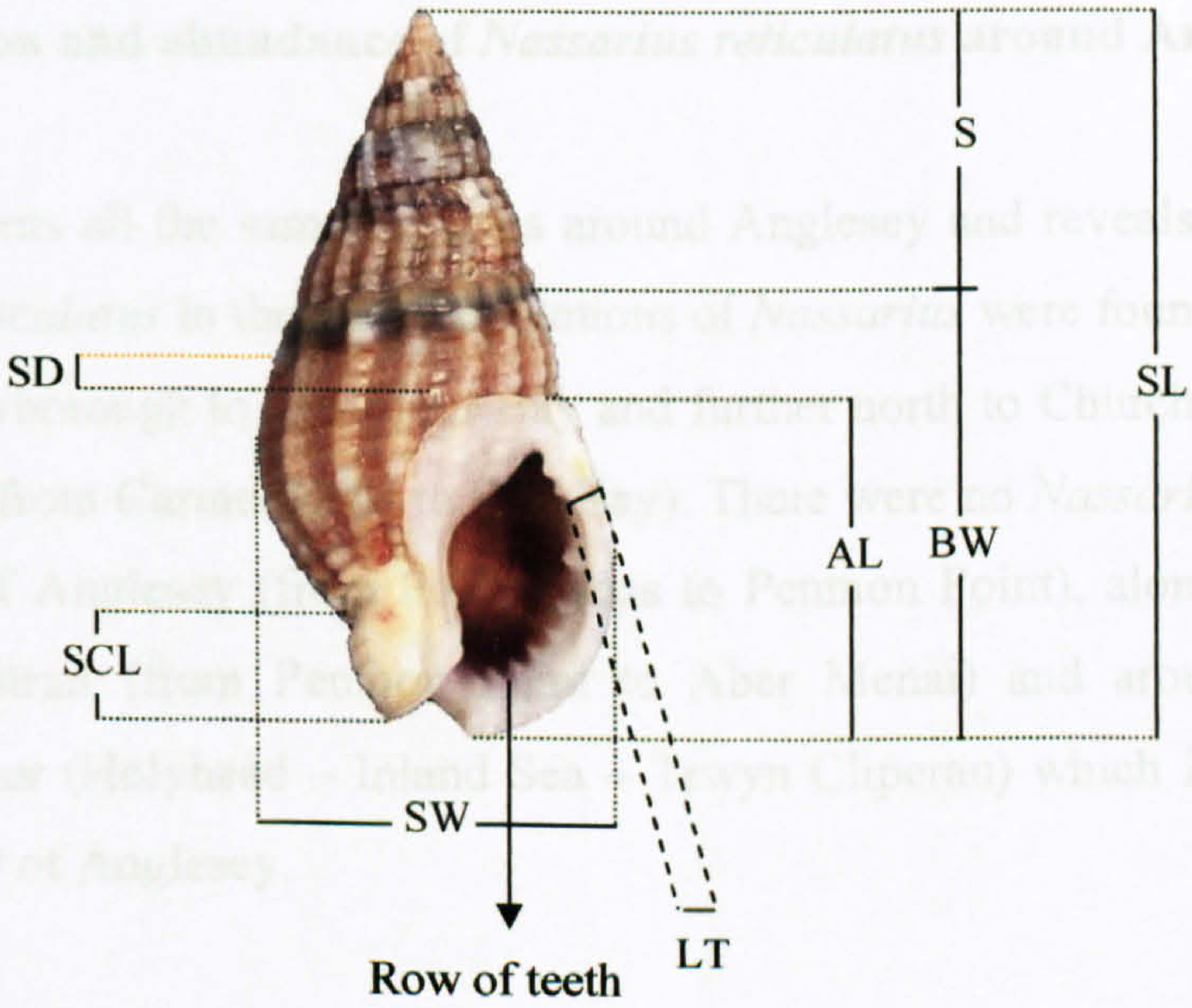
The median and mean grain size can be used to classify the sediment sample according to the Wentworth grade scale, and the standard deviation can be used to describe the sorting of the sample (Folk, 1968).

For the estimation of sediment organic content ~ 7.5 g of each sediment sample that had been previously stored in the desiccator was placed in a pre-weighed crucible. Initial dry weight of the sample was measured to the nearest 0.0001g. The crucibles containing the sediment samples were placed in an oven at 440°C temperature for 3 hours and then left inside the oven to cool down overnight. The weight of the sediment samples was measured again after ignition. The percentage difference in sediment weight before and after ignition represents the amount of organic material contained in each sample.

2.2.6 Morphological characteristics and allometric relationships

The morphological characteristics of the shells of *Nassarius reticulatus* in the sub-samples collected at each location were measured using a vernier calliper to the nearest 0.1 mm. Total shell length (SL), body whorl length (BW), shell width (SW), shell depth (SD), aperture length (AL), siphonal canal length (SCL) and lip thickness (LT) were measured (figure 2.5). The condition (thickness) of the shell lips of *N. reticulatus* was categorised as stage 1 (CL1) for animals with thin sharp shell lips, stage 2 (CL2) for animals with thickened lips, stage 3 (CL3) for animals with thick lips and a row of single teeth and finally stage 4 (CL4) for animals with thick lips and a wide row of elongated teeth (modified for *Nassarius* from Crothers, 1985a). The gender and possible imposex stage of *N. reticulatus* from all the sampling sites were identified and the results are presented in chapter 7.

Figure 2.5 Measurements of the morphological characteristics of the shell of *Nassarius reticulatus* used in the comparison of shell shape in gastropods collected from the different sampling areas. SL= shell length, S= spire length, BW= body whorl length, AL= aperture length, LT= lip thickness, SW= shell width, SCL= siphonal canal length, SD= shell depth.



In order to examine possible differential growth between the morphometric parameters measured, each pair of size variables was fitted to the allometric equation $Y = a X^b$ (Reiss, 1989). The allometric equation after logarithmic transformation takes the form of the expression: $\log Y = \log a + b \log X$, where Y is the dependent size variable, X is the independent size variable (shell length), b is the relative growth rate of the size variable (slope) and a is the initial growth coefficient (intercept). When the slope b is equal to 1 the relationship is isometric, when b is smaller than 1 the relationship is negatively allometric and when b is higher than 1 the relationship is positively allometric. The intercept a, the slope b and their standard errors were calculated by regression analysis between the two variable X and Y. A t-test was used in order to examine if the slope b was significantly different from unity ($\beta=1$), and thus to ascertain if the hypothesis $H_0=1$ for isometric relationship is accepted or not. The estimated t-value (t_s) was calculated from the equation $t_s= (b-\beta)/SE_b$, where b is the slope, SE_b is the standard error of the slope and β is the isometric value ($\beta=1$). When the estimated t-value (t_s) is lower than the tabulated t-value for a 95% level of significance (a=0.05) the $H_0=1$ hypothesis is accepted and the relationship is isometric. However, if the estimated t-value (t_s) is higher than the tabulated t-value the $H_0=1$ hypothesis is rejected and the relationship is allometric. A General Linear Model ANOVA analysis was used to compare the size variable relationships of the shells from the different sampling sites.

2.3 RESULTS

2.3.1 Distribution and abundance of *Nassarius reticulatus* around Anglesey

Figure 2.2 presents all the sampling sites around Anglesey and reveals the distribution of *Nassarius reticulatus* in the area. Populations of *Nassarius* were found along the west coast (from Newborough to Trearddur Bay and further north to Church Bay) and along the north coast (from Carmel Head to Bull Bay). There were no *Nassarius* present along the east coast of Anglesey (from Point Lynas to Penmon Point), along the south-east facing Menai Strait (from Penmon Point to Aber Menai) and around the area of Holyhead harbour (Holyhead – Inland Sea – Trwyn Cliperau) which is located on the north-west coast of Anglesey.

Population abundance was estimated as the mean number of *Nassarius reticulatus* collected per trap over a period of 30 minutes (table 2.2). High abundance of *Nassarius* was observed in the areas located on the west coast of Anglesey (Church Bay: 4.4 *Nassarius*·trap^{-1}, Cymyran Bay: 9.3 *Nassarius*·trap^{-1}, Rhosneigr: 13.6 *Nassarius*·trap^{-1}, Aberffraw: 20.6 *Nassarius*·trap^{-1}, Malltraeth: 23.4 *Nassarius*·trap^{-1}); Aberffraw and Malltraeth supported the highest population abundances. Low abundances of *N. reticulatus* were recorded in the south-western Newborough (0.4 *Nassarius*·trap^{-1}), and in Rhoscolyn (1.1 *Nassarius*·trap^{-1}) and Trearddur Bay (0.8 *Nassarius*·trap^{-1}) areas located on the south-west side of Holy Island. Populations of *N. reticulatus* along the north coast of Anglesey were also not very extensive (Carmel Head: 1.3 *Nassarius*·trap^{-1}, Cemlyn Bay: 1.5 *Nassarius*·trap^{-1}, Cemaes Bay: 0.4 *Nassarius*·trap^{-1} and Bull Bay: 1.3 *Nassarius*·trap^{-1}).

Table 2.2 The number of traps (n) deployed at each site for a period of 30 minutes, the total number of *Nassarius reticulatus* trapped and the number of *Nassarius* caught per trap at each site. The index of dispersion and the results of the chi-square test for n-1 degrees of freedom (a=0.05) are presented. n.a: not applicable

Sites	**Traps (n)**	**Total *Nassarius***	***Nassarius* ·trap^{-1}**	**Index of dispersion**	**Chi-square (DF=n-1, a=0.05)**
Newborough	10	4	0.4	2.33	21.00
Malltraeth	25	586	23.4	23.30	559.21
Aberffraw	30	618	20.6	21.67	628.54
Rhosneigr	20	271	13.6	28.38	539.22
Cymyran Bay	20	185	9.3	9.49	180.34
Rhoscolyn	7	8	1.1	3.62	21.74
Trearddur Bay	20	15	0.8	6.86	130.31
Holyhead	40	0	0	n.a	n.a
Inland Sea	10	0	0	n.a	n.a
Trwyn Cliperau	10	0	0	n.a	n.a
Church Bay	13	57	4.4	19.32	231.89
Carmel Head	8	10	1.3	10.03	70.18
Cemlyn Bay	20	29	1.5	10.35	196.55
Cemaes Bay	20	8	0.4	4.05	76.98
Bull Bay	10	13	1.3	4.45	40.08
Point Lynas	5	0	0	n.a	n.a
Dulas Bay	10	0	0	n.a	n.a
Moelfre	10	0	0	n.a	n.a
Red Wharf Bay	10	0	0	n.a	n.a
Penmon Point	15	0	0	n.a	n.a
Beaumaris	10	0	0	n.a	n.a
Bangor Pier	10	0	0	n.a	n.a
Menai Bridge	10	0	0	n.a	n.a
Tal-y-Foel	7	0	0	n.a	n.a
Traeth Melynog	8	0	0	n.a	n.a
Aber Menai	10	0	0	n.a	n.a

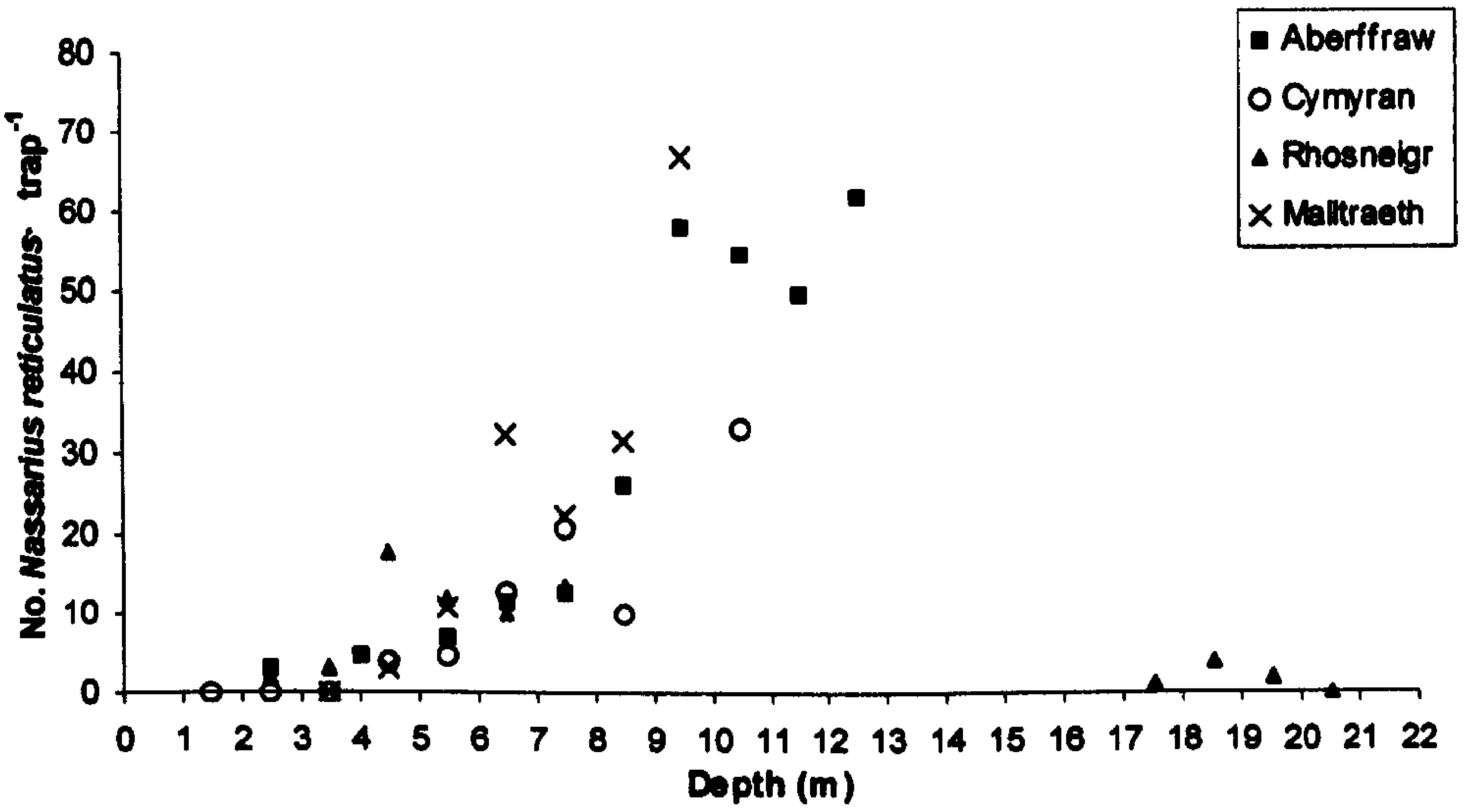
2.3.2 Index of dispersion

The indices of dispersion for all the sampling sites where *Nassarius reticulatus* were present are shown in table 2.2 and they were found to be significantly greatly than unity, indicating that the distribution of each population was clumped. The observed clumped distributions are probably due to the rapid aggregation of gastropods in areas where carrion sources occur. Immediately after feeding the gastropods burrow into the sediment to avoid predation and remain in the area where the carrion was found.

2.3.3 Bathymetric distribution

The bathymetric distribution of *Nassarius reticulatus* was studied only at Malltraeth, Aberffraw, Rhosneigr and Cymyran Bay where a high number of the gastropod was collected from several different depths in July 2003 (figure 2.6). The four sampling areas are subtidal sandy sites and sampling took place during high tide. The mean number of *Nassarius* collected per trap per 30-minute sampling period at stations of different depths is summarised in table 2.3. Data from a pilot survey undertaken at Rhosneigr in May 2002, during which a greater range of depths was covered (up to 21m depth) are also included.

Figure 2.6 Mean number of *Nassarius reticulatus* collected per 30 minutes per trap from different depths (m) at Aberffraw, Cymyran, Rhosneigr and Malltraeth.



A significant correlation (Pearson test $p<0.05$) was found between the abundance of gastropods and depth in Malltraeth ($r = 0.906$, $p=0.005$), Aberffraw ($r = 0.916$, $p<0.001$) and Cymyran ($r = 0.891$, $p=0.001$). In Rhosneigr the data are scattered with no significant correlation between number of gastropods·trap^{-1} and depth ($r= -0.564$, $p=0.090$), even after removing the data for depths >17m ($r= 0.641$, $p=0.170$). The number of *N. reticulatus* collected per trap was low in areas shallower than 4 m depth at all the sampling sites. The abundance of *Nassarius* increased with increasing depth, reaching a maximum at a depth of 9 to 10m in Malltraeth, 9 to 13m in Aberffraw and 10 to 11m in Cymyran. In Rhosneigr very few whelks were present at depths >17 m.

The mean shell size (±SE) and size range of whelks collected at different depths from the 4 sampling sites (Aberffraw, Rhosneigr, Cymyran and Malltraeth) are presented in table 2.3. Unfortunately no data were available for the size of whelks collected from Rhosneigr beach during the May 2002 pilot survey, where stations deeper than 7m were sampled. A wide range of shell sizes was present at all depths from the 4 sampling sites (table 2.3, figure 2.7). However, smaller sizes were observed at Aberffraw from a range of depths from 9 to 10m, in Rhosneigr from 6 to 7m depth (no data available for the deeper depth stations), in Cymyran from 6 to 8m and in Malltraeth from 6 to 8m (figure 2.7). At most sites large whelks were collected from the shallower and from the deeper depths.

Table 2.3 The number of *Nassarius reticulatus* caught per trap (N·trap^{-1}), mean shell length (mm) (±SE) and shell length range (min-max) (mm) of individuals collected from different depths (m) at Aberffraw, Rhosneigr, Cymyran and Malltraeth. n.a.: not available.

Aberffraw

Depth	N·trap^{-1}	Mean (±SE)	Range
2-3	3.0	20.8 (1.9)	16.0 – 26.3
3-5	4.5	19.8 (1.3)	12.2 – 27.7
6-5	7.0	19.3 (1.4)	11.0 – 26.9
6-7	11.5	20.4 (0.9)	11.7 – 29.6
7-8	12.6	18.5 (0.5)	6.9 – 28.6
8-9	26.0	18.0 (0.6)	10.5 – 28.9
9-10	58.0	16.9 (0.4)	9.9 – 26.9
10-11	54.5	18.7 (0.5)	10.2 – 28.9
11-12	49.5	18.7 (0.5)	11.0 – 29.0
12-13	62.0	19.3 (0.6)	11.8 – 29.2

Rhosneigr

Depth	N·trap^{-1}	Mean (±SE)	Range
2-3	2.0	15.5 (0.9)	13.5 - 17.7
3-4	3.0	16.2 (1.1)	12.6 - 18.8
4-5	17.6	15.7 (0.3)	7.8 – 27.8
5-6	12.0	17.9 (0.8)	8.4 – 26.8
6-7	10.1	13.9 (0.5)	8.8 – 23.7
7-8	13.5	n.a.	n.a.
17-18	1.0	n.a.	n.a.
18-19	4.0	n.a.	n.a.
19-20	2.0	n.a.	n.a.
20-21	0.0	-	-

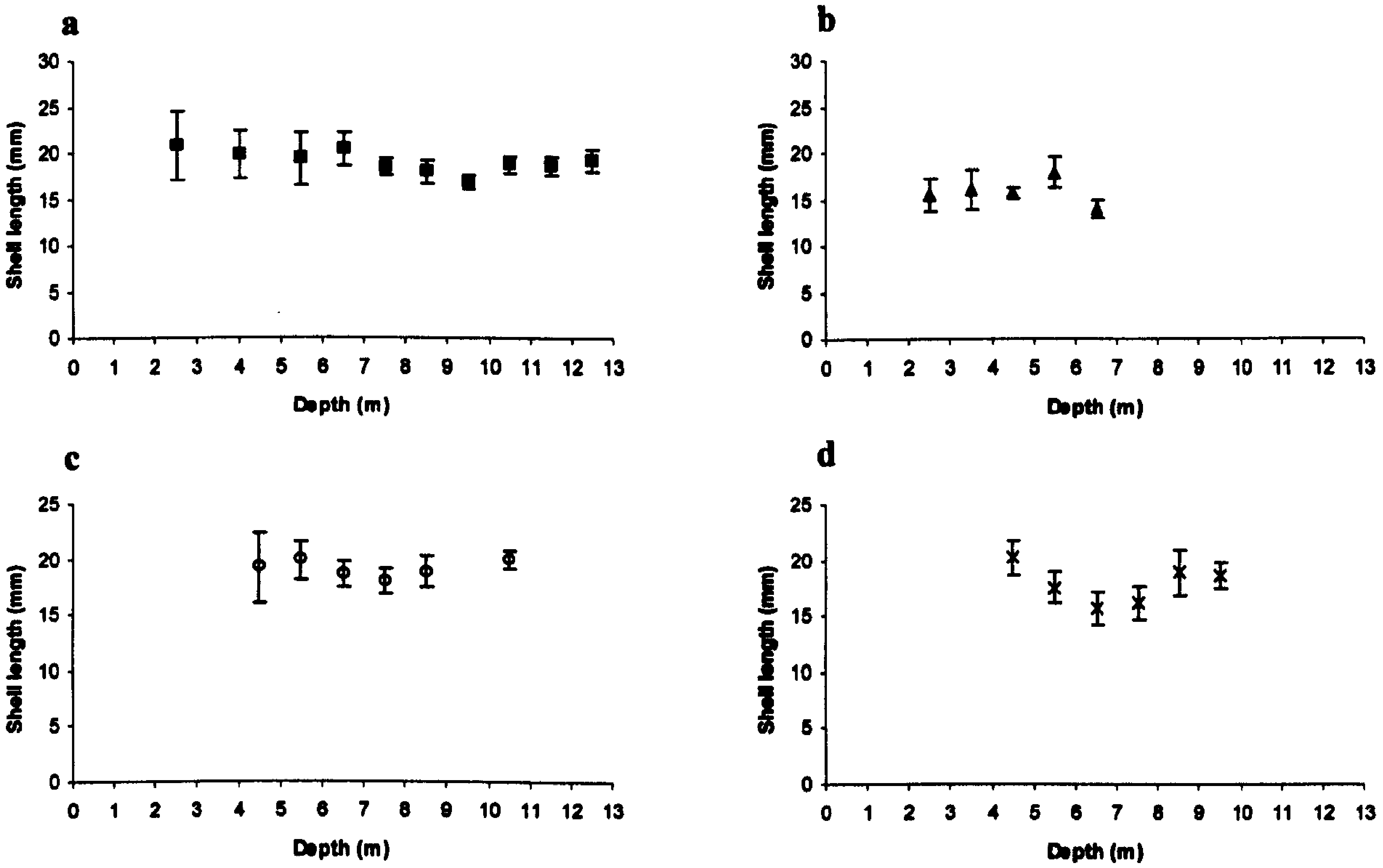
Malltraeth

Depth	N·trap^{-1}	Mean (±SE)	Range
1-2	0.0	-	-
2-3	0.0	-	-
3-4	0.0	-	-
4-5	3.8	20.2 (0.8)	16.3 – 22.4
5-6	4.5	17.5 (0.7)	11.4 – 23.5
6-7	12.8	15.7 (0.7)	9.2 – 25.7
7-8	20.8	16.2 (0.7)	6.3 – 29.8
8-9	10.0	18.9 (1.0)	9.4 – 29.6
9-10	33.0	18.6 (0.5)	9.8 – 31.2

Cymyran

Depth	N·trap^{-1}	Mean (±SE)	Range
3-4	0.0	-	-
4-5	3.2	19.2 (1.6)	10.5 - 28.2
5-6	10.8	19.9 (0.9)	11.1 - 25.1
6-7	32.2	18.6 (0.6)	11.6 - 26.7
7-8	22.5	18.0 (0.6)	10.2 - 27.3
8-9	31.5	18.9 (0.7)	11.4 - 24.4
10-11	67.0	20.0 (0.4)	15.5 - 25.2

Figure 2.7 Bathymetric distribution of shell length (mm) of *Nassarius reticulatus* collected from different depths (m) at a) Aberffraw (■), b) Rhosneigr (▲), c) Cymyran (o) and d) Malltraeth (x).The error bars represent the 95% confidence intervals (1.96· SE) of the means.



The median shell length of gastropods from the four sampling sites was compared between different depths using non-parametric tests (non-normal data). The Kruskal-Wallis test was used when the data had equal variances and the Mood's Median test was used in cases for data with non-equal variances. There was a significant difference in the median shell length between different depths at Aberffraw (Kruskal-Wallis: H=24.78, p=0.003), Rhosneigr (Mood's Median: Chi-Sq=12.28, p=0.015) and Malltraeth (Mood's Median: Chi-Sq=11.33, p=0.045), where gastropods collected from 9-10m, 6-7m and 7-8m depths respectively were significantly smaller than at the other depths. However, there was no significant difference in the median shell length between the different depths at Cymyran Bay (Mood's Median: Chi-Sq=6.12, p=0.295).

2.3.4 Predators

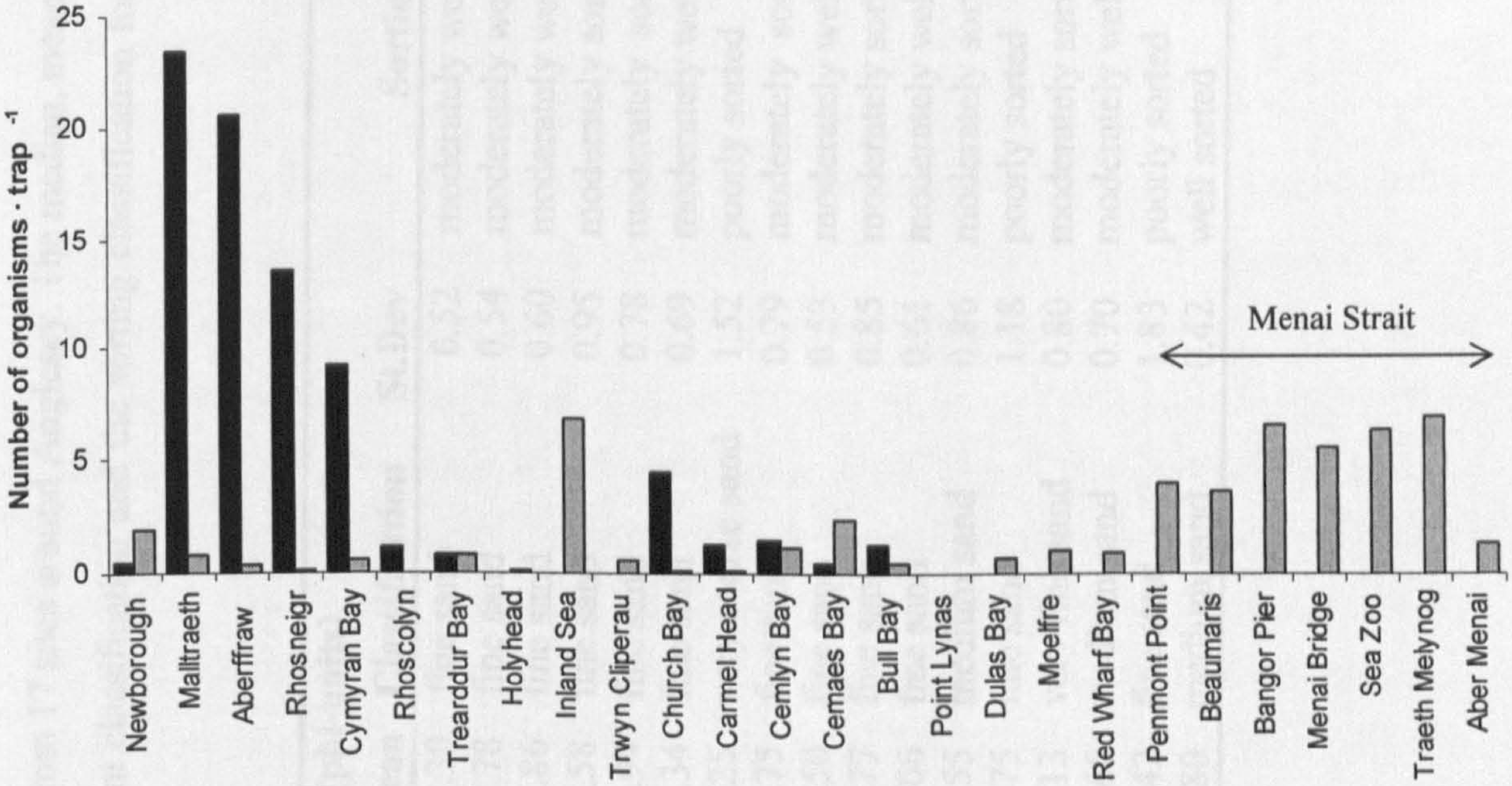
Several organisms were attracted to the baited traps during the collection of the *Nassarius reticulatus* (see table 2.4). Crabs and starfish are common predators of

Table 2.4 Number of traps deployed at each site for a period of 30 minutes and the total number of crab and other species caught at each site. The mean number of predators per trap includes all crab species (except hermit crabs) and the starfish *Astropecten irregularis*. Shore crab= *Carcinus maenas*, swimming crab= *Liocarcinus holsatus*, velvet swimming crab= *Necora puber*, spider crab= *Maja squinado*, edible crab= *Cancer pagurus*, hermit crab= *Pagurus bernhardus*.

Sites	No of traps	Shore crabs	Swimming crabs	Velvet swimming crabs	Spider crabs	Edible crabs	Hermit crabs	Predators $.trap^{-1}$	Other species
Newborough	10	19					1	1.9	
Malltraeth	25	4	7		5	3	23	0.8	
Aberffraw	30		6		3		44	0.3	
Rhosneigr	20		1		1		74	0.1	
Cymyran Bay	20	3	7				84	0.6	2 *Astropecten irregularis*
Rhoscolyn	7						6	0.0	
Trearddur Bay	20	8	7		1		19	0.8	
Holyhead	40		1	1	1	1		0.1	1 *Homarus gammarus*
Inland Sea	10	65	2		1		2	6.8	1 *Crangon crangon*
Trwyn Cliperau	10	4	1			1	9	0.6	
Church Bay	13				1			0.1	
Carmel Head	8						1	0.1	1 *Astropecten irregularis*
Cemlyn Bay	20	16		2	1		2	1.1	3 *Astropecten irregularis* 25 *Ophiura ophiura* 1 *Palaemon adspersus*
Cemaes Bay	20	44	3				1	2.4	
Bull Bay	10	5					3	0.5	
Point Lynas	5							0.0	
Dulas Bay	10	6		1				0.7	10 *Ophiura ophiura*
Moelfre	10	3	4	2			1	1.0	7 *Ophiura ophiura* 1 *Astropecten irregularis*
Red Wharf Bay	10	1	6		2			0.9	8 *Ophiura ophiura* 3 *Neptunea antiqua*
Penmon Point	15	56	2					4.0	2 *Astropecten irregularis* 1 *Crangon crangon*
Beaumaris	10	36					1	3.7	1 *Astropecten irregularis*
Bangor Pier	10	66						6.6	42 *Ophiura ophiura*
Menai Bridge	10	56						5.6	
Tal-y-Foel	7	45					4	6.4	
Traeth Melynog	8	56						7.0	3 *Crangon crangon*
Aber Menai	10	14						1.4	1 *Crangon crangon*

N. reticulatus (Gore, 1966; Stenzler & Atema, 1977; Fretter & Graham, 1994). Cases of hermit crabs attacking gastropods have been rarely observed, and where they have been seen this has involved a few large aggressive hermit crab species (Vermeij, 1978), which were not present in the current study. More predators were observed in the Menai Strait and in the Inland Sea (figure 2.8). No *N. reticulatus* were present at either of these sites where predator species were abundant. The number of predators was low at all the sampling sites where there was a high abundance of *N. reticulatus* as seen on the west coast of Anglesey.

Figure 2.8 Numbers of *Nassarius reticulatus* (black bars) and predators (crabs and starfish species) (grey bars) caught per trap per 30 minutes from each site.



2.3.5 Sediment analysis

The median, mean and standard deviation of grain size from each site are presented in table 2.5. The mean grain size used for sediment classification is based on the Wentworth scale and the standard deviation was used for describing the degree of sorting (uniformity) of the sediment (Folk, 1968). The percentage of silt content in each sediment sample was calculated from the weight of sediment <63 um. The % silt content, together with the results of the organic content analysis are also summarised in table 2.5.

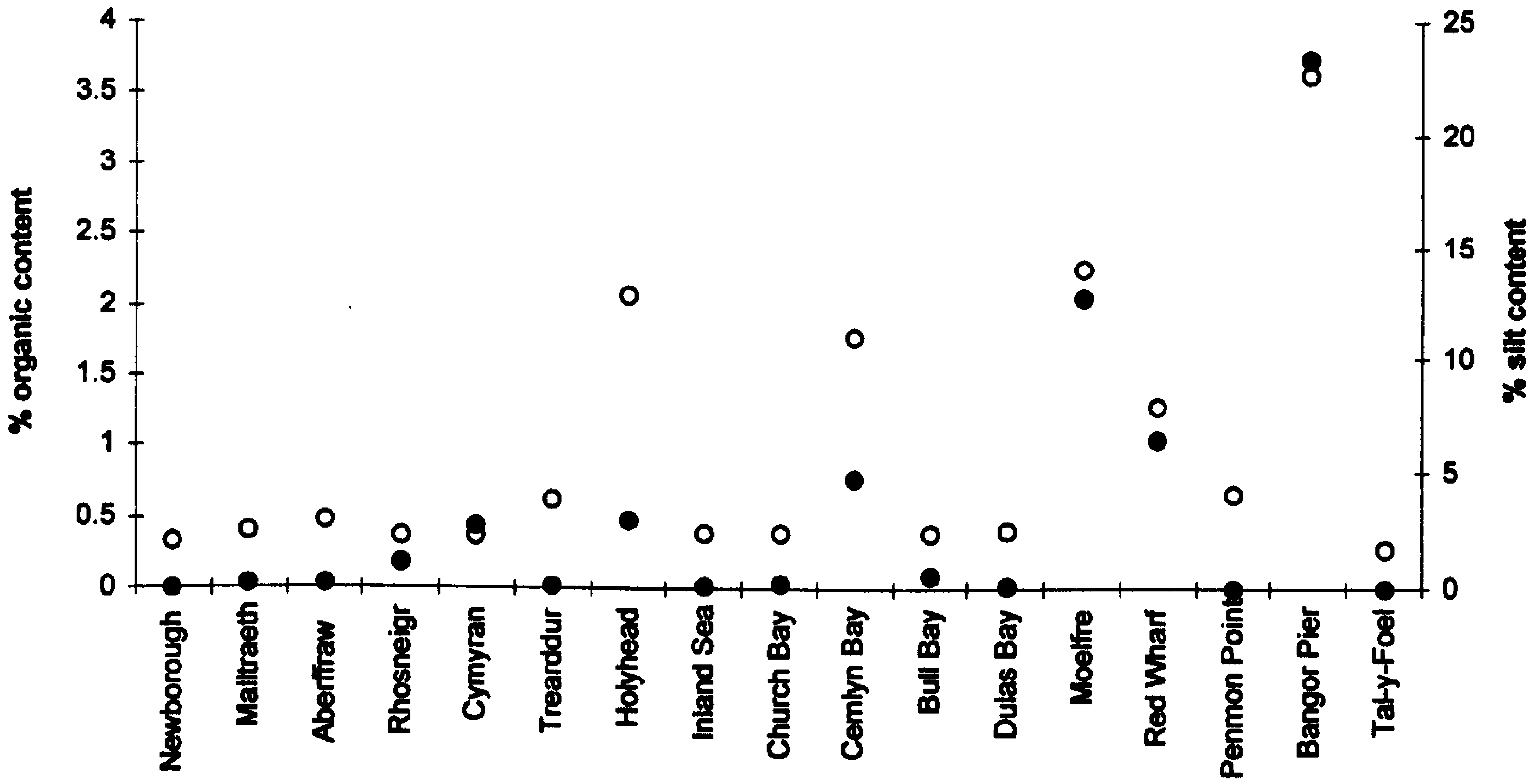
Table 2.5 Results of the analysis of sediments from 17 sites around Anglesey. The median, mean and standard deviation of grain size (phi units) are presented together with the Wentworth sediment classification and the sorting classification for all sediment samples. The percentage of silt and organic content for each site are also shown.

Sites	Grain size Ø (phi-units)					% silt content	% organic content
	Median	Mean	Classification	St.Dev	Sorting		
Newborough	2.32	2.30	fine sand	0.52	moderately well sorted	0.030	0.330
Malltraeth	2.79	2.78	fine sand	0.54	moderately well sorted	0.199	0.407
Aberffraw	2.89	2.86	fine sand	0.60	moderately well sorted	0.221	0.476
Rhosneigr	2.84	2.58	fine sand	0.95	moderately sorted	1.132	0.363
Cymyran Bay	3.00	2.94	fine sand	0.78	moderately sorted	2.710	0.364
Trearddur Bay	2.43	2.34	fine sand	0.69	moderately well sorted	0.110	0.615
Holyhead	-1.58	-0.25	very coarse sand	1.52	poorly sorted	2.987	2.063
Inland Sea	2.84	2.75	fine sand	0.79	moderately sorted	0.123	0.375
Church Bay	2.51	2.50	fine sand	0.53	moderately well sorted	0.179	0.387
Cemlyn Bay	2.79	2.77	fine sand	0.85	moderately sorted	4.787	1.767
Bull Bay	2.12	2.06	fine sand	0.61	moderately well sorted	0.617	0.388
Dulas Bay	1.74	1.55	medium sand	0.86	moderately sorted	0.133	0.408
Moelfre	2.89	2.75	fine sand	1.18	poorly sorted	12.780	2.246
Red Wharf Bay	3.18	3.13	very fine sand	0.80	moderately sorted	6.472	1.280
Penmon Point	1.79	1.65	medium sand	0.70	moderately well sorted	0.010	0.658
Bangor Pier	2.89	2.42	fine sand	1.83	poorly sorted	23.455	3.630
Tal-y-Foel	1.84	1.80	medium sand	0.42	well sorted	0.010	0.265

All the sampling sites along the west coast of Anglesey, from Newborough in the south to Church Bay in the north, are characterised by moderately sorted fine sand sediments. The exception was Holyhead harbour sediment, which was classified as poorly sorted very coarse sand. Along the north coast, Cemlyn Bay and Bull Bay, were classified as moderately sorted fine sand sediments. A variation in sediment types was present along the east coast of Anglesey, from moderately sorted medium sand at Dulas Bay and Penmon Point, poorly sorted fine sand at Moelfre and moderately sorted very fine sand in Red Wharf Bay. The subtidal sites in the Menai Strait were characterised by poorly sorted fine sand at Bangor Pier, and well sorted medium sand in Tal-y-Foel. Following the above results it can be concluded that *Nassarius reticulatus* was present in sites where the sediment was moderately sorted fine sand. No *Nassarius* were found where the sediment was very coarse, medium or very fine sand or where the sediment was fine sand but poorly sorted.

Sediment samples rich in silt content were also the ones with the highest organic content (figure 2.9). Organic content was lower than 0.5 % in most of the west and north coast sites, and only the sediment collected from Holyhead harbour and Cemlyn Bay (near the nuclear Power Plant) had a high organic content of 2.06 % and 1.77 % respectively. Accordingly, the percentage of silt content was lower than 2 % in most of the west and north coast sites and only the sediment collected from Cymyran Bay, Holyhead harbour and Cemlyn Bay had a higher silt content of 2.71 %, 2.99 % and 4.79 % respectively. Moelfre, Red Wharf Bay and Penmon Point, located along the east coast of Anglesey, were richer in organic material (2.25%, 1.28% and 0.66 % respectively) than the north-west areas. The percentage of silt content was also higher at the eastern sites with 12.78% in Moelfre and 6.47% in Red Wharf Bay. The silt content in sediments from Penmon Point was low (0.01%). The Bangor Pier site, situated at the eastern entrance to the Menai Strait, had the highest organic and silt content (3.63% and 23.46% respectively) of all sites examined, while at Tal-y-Foel, located towards the western entrance to the Menai Strait, the sediment contained the lowest organic and silt content (0.27% and 0.01 respectively) of all sites. The above results indicate that the west and north Anglesey sites, where *Nassarius reticulatus* was present, were characterised by a low silt and organic content.

Figure 2.9 Percentage of organic (o) and silt (•) content in sediment samples collected from different Anglesey sites.



The mean number of animals collected per trap from all sites was correlated with the 3 sediment parameters mean grain size, percentage silt content and percentage organic content (table 2.6). A high abundance of *Nassarius reticulatus* was found in sediments where the silt content ranged between 0.2 and 3%, while in sediments where the silt content was <0.2% or >3% the mean number of animals collected per trap was low. The mean number of animals per trap was higher in sediments with a range of organic content from 0.36-0.39% and 0.4-0.5 % (5.7 and 14.7 animals per trap respectively), while in areas with sediments rich in organic content (>2%) no whelks were collected. The highest abundance of *N. reticulatus* (22 animals per trap) was found in fine sand (2.78 to 2.89 phi units).

Table 2.6 Mean number of *Nassarius reticulatus* caught per trap for each of the 7 classes of silt content, organic content and grain size (phi units).

% silt content	<0.1	0.1-0.19	0.2-0.9	1-1.99	2-2.99	3-4.99	>5
Nassarius.trap^{-1}	0.1	0.3	12.4	13.6	9.3	0.8	0.0
% organic content	<0.30	0.30-0.35	0.36-0.39	0.4-0.5	0.6-0.7	0.8-2	>2
Nassarius.trap^{-1}	0	0.4	5.7	14.7	0.4	0.8	0.0
Mean grain size Ø	<0	0-1.9	2-2.4	2.5-2.77	2.78-2.89	2.90-3	>3
Nassarius.trap^{-1}	0	0.0	0.6	3.9	22.0	9.3	0.0

2.3.6 Morphological characteristics of the *Nassarius reticulatus* shell and allometric relationships

The morphological shell characters of *Nassarius reticulatus* collected from the different sampling sites around Anglesey are summarised in table 2.7. When comparisons between size variables were made using ANOVA analysis a significant difference was found in shell length (F=11.26, p<0.001), whorl length (F= 10.97, p<0.001), aperture length (F=17.04, p<0.001), shell width (F=10.42, p<0.001), shell depth (F= 10.47, p<0.001), siphonal canal length (F=9.80, p<0.001) and thickness of lips (F=11.84, p<0.001) between the sampling sites. *Nassarius* from Church Bay, a wave-exposed site, had the smallest shell length, whorl length, aperture length, siphonal canal length, as well as shell depth, shell width and shell thickness.

Upon a further more detailed examination, there were no significant differences in shell length (F=1.30, p=0.250), whorl length (F=0.99, p=0.436), shell width (F=1.52, p=0.158), shell depth (F=1.79, p=0.088) and siphonal canal length (F=1.48, p=0.171) between all the sheltered and two of the semi-wave-exposed areas (Aberffraw, Rhosneigr, Cymyran, Rhoscolyn, Trearddur, Carmel-Cemlyn, Cemaes, Bull Bay). However, these variables were significantly larger in the semi-wave-exposed Malltraeth site, and significantly smaller in the wave-exposed Church Bay site. Similarly, the aperture length was not significantly different in whelks between the sheltered sites of Rhosneigr, Cymyran, Rhoscolyn, Trearddur, Carmel-Cemlyn, Bull Bay and the semi-wave-exposed site Cemaes Bay (F=1.91, p=0.079). However, aperture length was significantly larger in whelks from the semi-wave-exposed sites of Malltraeth and Aberffraw and significantly smaller from the wave-exposed Church Bay site. Greater inter-site variation was observed regarding the thickness of the shell lips, and similarities were noticed only within the following sites: a) Trearddur Bay and Rhoscolyn (F=0.31, p=0.581), b) Cymyran Bay, Aberffraw and Malltraeth (F= 2.38, p=0.094) and c) Rhosneigr, Carmel-Cemlyn, Cemaes, Bull Bay (F=1.63, p=0.185). Whelks from the wave-exposed site of Church Bay had significantly thinner shell lips than at all the other sites.

Shell length was used in all allometric regressions as the independent variable (X) and whorl length, aperture length, shell width, shell depth, length of siphonal canal and

Table 2.7 Mean size in mm (±SE) and range (min – max) of 8 morphological shell dimensions of *Nassarius reticulatus* from 10 sites around Anglesey. N: number of gastropods measured from each site.

Sampling sites	N	Shell length			Whorl length			Aperture length			Shell width		
		mean	±SE	range	mean	±SE	range	mean	±SE	range	mean	±SE	range
Malltraeth	63	22.9	±0.5	12.3 – 31.4	16.0	±0.3	8.8 –20.2	11.6	±0.2	6.6 – 14.7	10.7	±0.2	5.9 – 13.3
Aberffraw	111	21.2	±0.3	13.5 – 29.0	15.0	±0.2	10.0 – 19.8	11.0	±0.2	7.6 – 15.5	10.0	±0.2	6.7 – 13.4
Rhosneigr	86	20.9	±0.3	15.6 – 27.8	14.9	±0.2	11.5 – 19.5	10.5	±0.1	8.0 – 14.3	10.2	±0.1	7.8 – 12.7
Cymyran Bay	108	20.7	±0.3	16.0 – 28.2	14.8	±0.2	11.4 – 19.1	10.4	±0.1	8.0 – 13.9	10.1	±0.1	7.9 – 13.7
Rhoscolyn	8	21.0	±1.7	11.6 – 25.7	14.6	±1.0	8.70 – 17.8	9.6	±0.7	5.3 – 11.9	10.8	±0.8	6.8 – 13.4
Trearddur Bay	15	22.2	±0.8	15.8 – 26.4	15.5	±0.6	11.3 – 18.6	10.6	±0.4	7.6 – 13.3	10.9	±0.3	7.5 – 12.8
Church Bay	46	16.9	±0.3	11.4 – 21.1	12.2	±0.2	8.2 – 15.2	8.6	±0.1	6.3 – 10.3	8.3	±0.2	5.6 – 10.3
Carmel-Cemlyn	39	20.4	±0.5	14.6 – 26.2	14.4	±0.3	10.6 – 18.0	10.4	±0.2	8.0 – 12.7	10.2	±0.2	7.8 – 12.7
Cemaes Bay	10	18.9	±1.1	13.4 – 24.5	13.8	±0.7	9.9 – 16.7	9.5	±0.5	7.3 – 12.0	9.8	±0.5	7.0 – 11.5
Bull Bay	13	21.0	±0.9	15.4 – 26.2	14.7	±0.6	11.3 – 18.0	10.1	±0.4	7.8 – 12.3	10.9	±0.4	8.0 – 13.2

Sampling sites	N	Shell depth			Siph. canal length			Lip thickness		
		mean	±SE	range	mean	±SE	range	mean	±SE	range
Malltraeth	63	9.8	±0.2	5.3 – 12.2	3.3	±0.09	1.6 – 4.9	0.4	±0.02	0.1 – 0.9
Aberffraw	111	9.1	±0.1	6.0 – 12.3	3.0	±0.06	1.7 – 4.4	0.3	±0.02	0.1 – 1.0
Rhosneigr	86	9.2	±0.1	7.0 – 11.6	3.1	±0.04	2.3 – 4.2	0.4	±0.02	0.2 – 1.1
Cymyran Bay	108	9.4	±0.1	6.9 – 13.0	3.1	±0.06	2.2 – 4.7	0.3	±0.02	0.1 – 1.0
Rhoscolyn	8	9.6	±0.8	5.3 – 12.2	3.1	±0.26	1.7 – 3.8	0.6	±0.12	0.1 – 1.1
Trearddur Bay	15	9.9	±0.3	7.0 – 12.0	3.1	±0.12	2.2 – 4.2	0.7	±0.05	0.3 – 0.9
Church Bay	46	7.5	±0.1	5.1 – 9.2	2.4	±0.06	1.2 – 2.9	0.3	±0.02	0.1 – 0.5
Carmel-Cemlyn	39	9.2	±0.2	7.0 – 11.6	3.2	±0.08	2.0 – 4.4	0.4	±0.02	0.2 – 0.7
Cemaes Bay	10	8.7	±0.4	6.6 – 10.8	2.7	±0.19	1.8 – 3.3	0.3	±0.03	0.2 – 0.5
Bull Bay	13	10.1	±0.4	7.5 – 12.3	3.1	±0.13	2.5 – 3.7	0.5	±0.06	0.2 – 1.0

thickness of lips were used as the dependent variables (Y) (figure 2.10). A significant correlation ($p<0.05$) was found between the variables X and Y for all sampling sites. All the data were log transformed in order to determine their fit to the logarithmic form of the allometric equation ($\log Y = \log a + b \operatorname{Log} Y$). The normality and equal variance criteria for linear regressions were met for all data sets. The estimated intercept (a) and slope (b) from the regression analysis between variables X and Y and the calculated t-values (t_s) for determination of the allometric relationships are shown in table 2.8. A negative allometric relationship indicates that the relative growth of the size variable examined is slower than shell length growth, while a positive allometric relationship indicates faster growth of the size variable relative to shell length. An isometric relationship indicates similar growth between the size variable and shell length.

The relationship between shell length and whorl length was negatively allometric for whelks from Malltraeth, Aberffraw, Rhosneigr, Cymyran Bay, Rhoscolyn, Church Bay and Bull Bay, indicating that shell length is increasing at a relative faster rate than whorl length. However, at Trearddur Bay, Carmel–Cemlyn and Cemaes Bay the relationship was isometric, thus the whorl length and the shell length are increasing at the same relative rate. Similarly, the relationship between shell length and aperture length was negatively allometric for all the sites examined, with the exception of Rhoscolyn, Trearddur Bay and Cemaes Bay sites where the relationship was isometric. The relationship between shell length and siphonal canal length was positively allometric for Malltraeth and Aberffraw, negatively allometric for Rhosneigr, and isometric for the remaining sites.

The relationship between shell length and shell width was negatively allometric for *Nassarius reticulatus* from Malltraeth, Aberffraw, Rhosneigr, Cymyran, Rhoscolyn and Carmel- Cemlyn, while for Trearddur, Church Bay, Cemaes and Bull Bay an isometric relationship was found. Accordingly, the relationship between shell length and shell depth was negatively allometric for Malltraeth, Aberffraw, Rhosneigr, Carmel-Cemlyn and Cemaes, although for Cymyran, Rhoscolyn and, Trearddur, Church Bay and Bull Bay an isometric relationship was found. The negative allometric relationship of shell length with both shell width and shell depth in whelks from Malltraeth, Aberffraw Rhosneigr and Carmel-Cemlyn indicates that shell length increased faster than shell

Figure 2.10 Relationships between various shell dimensions in *Nassarius reticulatus* from 10 sites around Anglesey. (a) shell length and whorl length, (b) shell length and aperture length, (c) shell length and shell width, (d) shell length and shell depth, (e) shell length and siphonal canal length, (f) shell length and thickness of lips: Malltraeth (×), Aberffraw (▪), Rhosneigr (□), Cymyran Bay (△), Rhoscolyn (•), Trearddur Bay (•), Church Bay (+), Carmel–Cemlyn (○), Cemaes Bay (–) and Bull Bay (▲).

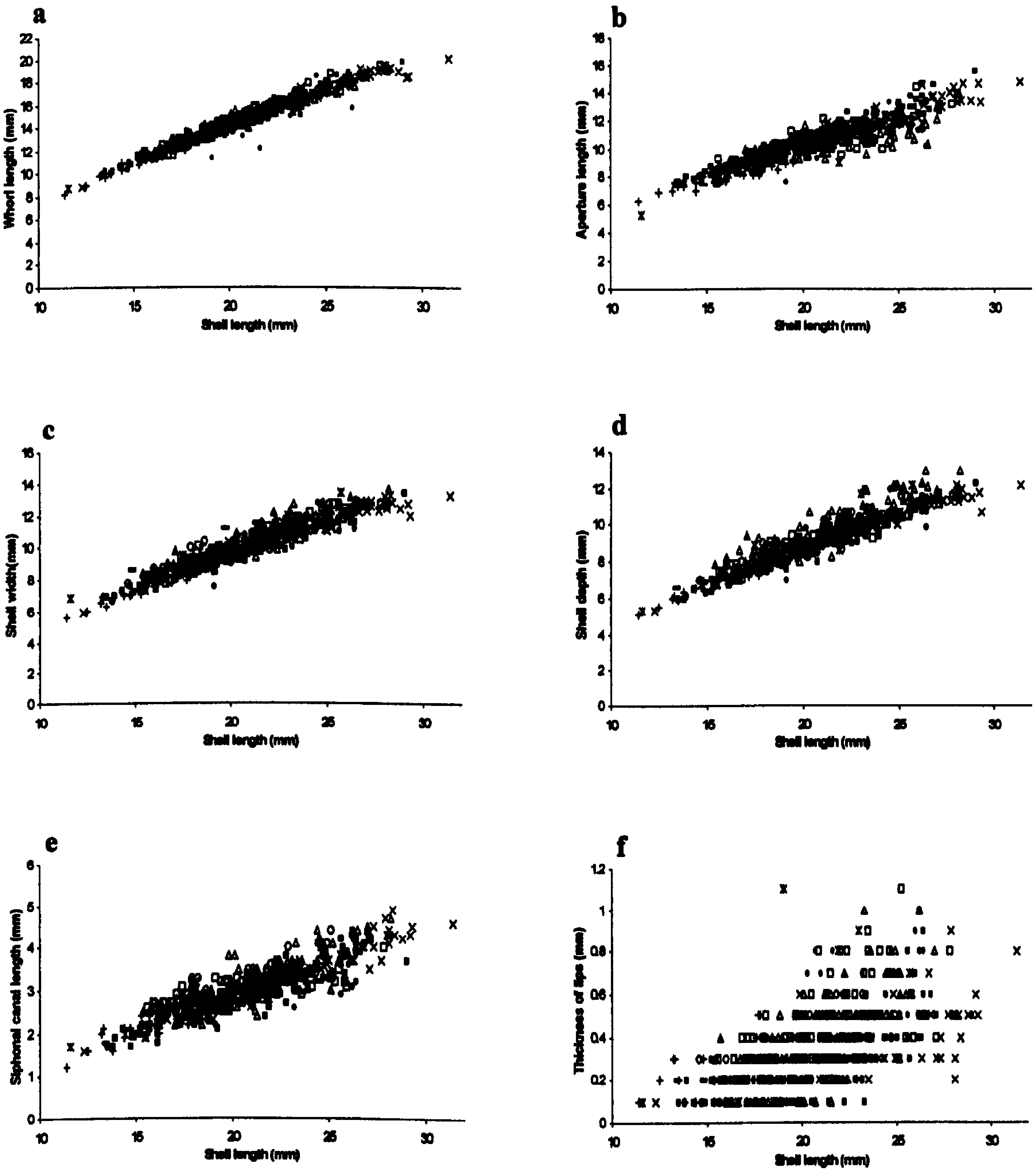


Table 2.8 Regression coefficients a (intercept) and b (slope) between morphological variables X and Y for all the sampling sites. The estimated t-value (t_s) and degrees of freedom (DF) are also shown. (Sip.Can: Siphonal canal, Thic.lip: lip thickness)

X – Y variables	**Malltraeth** (DF= 62)				**Aberffraw** (DF= 110)			
	a	b	ts		a	b	ts	
Length – Whorl	1.018	0.881	-7.532	allometric -	0.947	0.905	-7.111	allometric -
Length – Aperture	0.780	0.862	-5.151	allometric -	0.835	0.845	-7.666	allometric -
Length – Width	0.741	0.852	-6.795	allometric -	0.622	0.909	-5.401	allometric -
Length – Depth	0.634	0.873	-6.144	allometric -	0.518	0.939	-3.015	allometric -
Length – Sip.can.	0.096	1.130	2.761	allometric +	0.069	1.230	5.191	allometric +
Length – Thic.lip	$5\cdot10^{-4}$	2.060	4.579	allometric +	$4.3\cdot10^{-5}$	2.830	7.777	allometric +

X – Y variables	**Rhosneigr** (DF= 85)				**Cymyran Bay** (DF= 107)			
	a	b	ts		a	b	ts	
Length – Whorl	0.907	0.920	-3.852	allometric -	1.068	0.868	-8.435	allometric -
Length – Aperture	1.538	0.633	-8.152	allometric -	1.862	0.569	-9.858	allometric -
Length – Width	0.780	0.845	-5.401	allometric -	0.681	0.892	-3.850	allometric -
Length – Depth	0.643	0.876	-4.255	allometric -	0.471	0.987	-0.307	isometric
Length – Sip.can.	0.394	0.677	-5.710	allometric -	0.104	1.110	1.554	isometric
Length – Thic.lip	$9.8\cdot10^{-4}$	1.970	3.399	allometric +	$4.3\cdot10^{-6}$	3.660	7.461	allometric +

X – Y variables	**Rhoscolyn** (DF= 7)				**Trearddur Bay** (DF= 14)			
	a	b	ts		a	b	ts	
Length – Whorl	1.039	0.869	-3.804	allometric -	0.746	0.977	-0.278	isometric
Length – Aperture	0.545	0.943	-0.643	isometric	0.687	0.882	-1.104	isometric
Length – Width	0.961	0.794	-2.913	allometric -	0.783	0.848	-1.459	isometric
Length – Depth	0.509	0.963	-0.280	isometric	0.541	0.937	-1.046	isometric
Length – Sip.can.	0.157	0.976	-0.206	isometric	0.228	0.843	-0.910	isometric
Length – Thic.lip	$7.2\cdot10^{-4}$	2.160	1.443	isometric	0.005	1.570	1.112	isometric

X – Y variables	**Church Bay** (DF= 45)				**Carmel - Cemlyn** (DF=38)			
	a	b	ts		a	b	ts	
Length – Whorl	0.832	0.949	-2.190	allometric -	0.816	0.952	-1.564	isometric
Length – Aperture	1.175	0.703	-5.811	allometric -	0.922	0.802	-4.717	allometric -
Length – Width	0.457	1.020	0.459	isometric	0.990	0.773	-4.481	allometric -
Length – Depth	0.520	0.944	-1.679	isometric	0.911	0.768	-4.311	allometric -
Length – Sip.can.	0.102	1.110	1.126	isometric	0.132	1.060	0.772	isometric
Length – Thic.lip	0.002	1.710	1.670	isometric	0.002	1.710	2.846	allometric +

X – Y variables	**Cemaes Bay** (DF= 9)				**Bull Bay** (DF=12)			
	a	b	ts		a	B	ts	
Length – Whorl	1.042	0.878	-2.058	isometric	1.183	0.828	-3.405	allometric -
Length – Aperture	0.655	0.910	-0.908	isometric	1.056	0.743	-3.399	allometric -
Length – Width	0.832	0.839	-1.400	isometric	0.810	0.853	-2.105	isometric
Length – Depth	0.738	0.838	-2.440	allometric -	0.764	0.848	-2.076	isometric
Length – Sip.can.	0.098	1.130	0.704	isometric	0.327	0.736	-1.966	isometric
Length – Thic.lip	0.003	1.640	2.647	allometric +	0.005	1.500	0.792	isometric

width and depth resulting in more elongated shells. The relationship between shell length and thickness of lips was positively allometric for Malltraeth, Aberffraw, Rhosneigr, Cymyran, Carmel-Cemlyn and Cemaes whelks, indicating that thickening of the shell lips is faster than the increase in shell length. However, in Rhoscolyn, Trearddur, Church Bay and Bull Bay the relationship was isometric. These results suggest that there are differences in the way the shell of *Nassarius reticulatus* is growing between the different sampling sites.

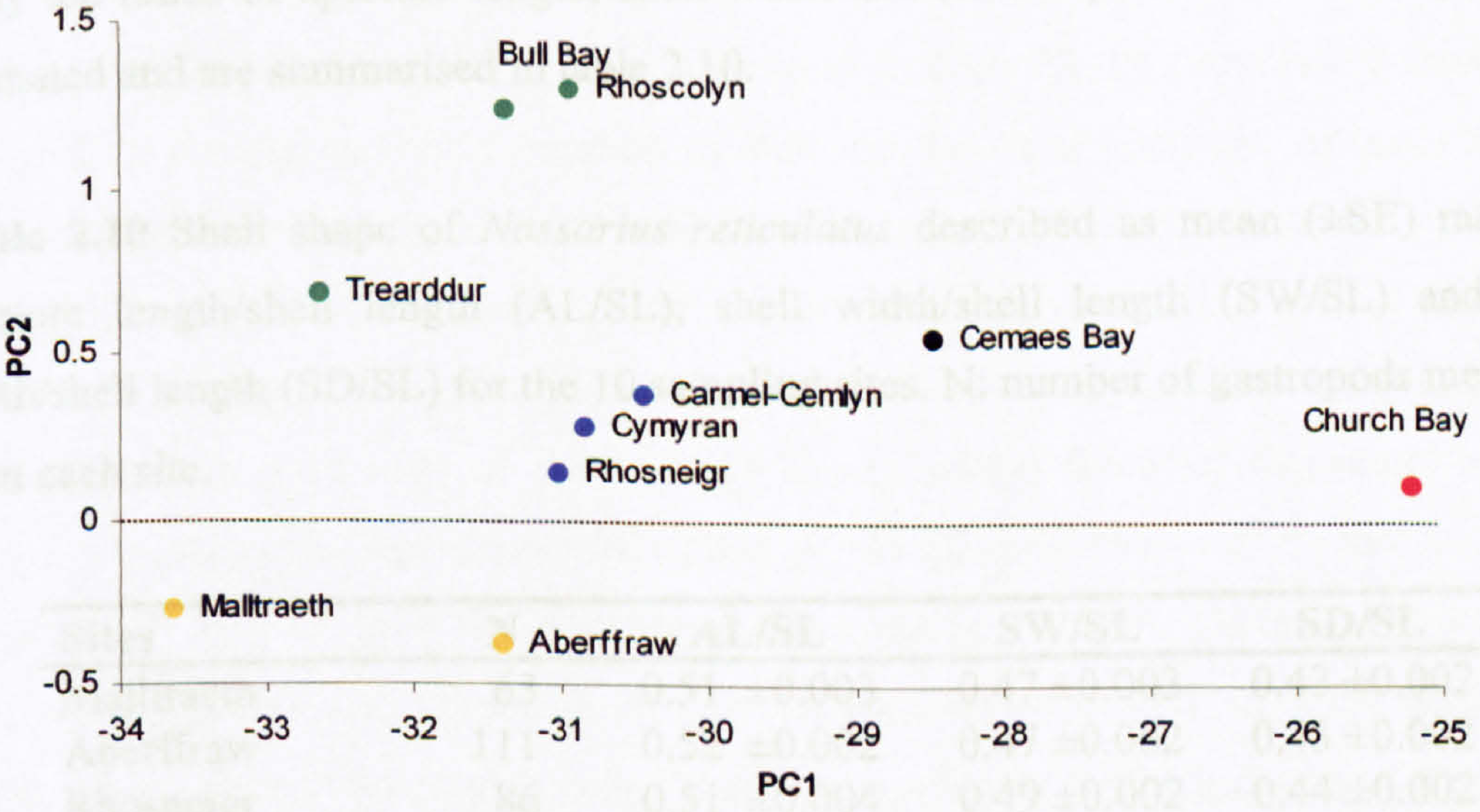
A General Linear Model, ANOVA comparison, confirmed that both the intercept and the slope of all the relationships were significantly different between the sampling sites (table 2.9). In order to clarify where there were similarities and dissimilarities in the relationships between the shell dimensions of *Nassarius reticulatus* from the various sampling sites a Principal Component Analysis (PCA) was performed. The PCA included the 7 size variables of the *N. reticulatus* shell and was based on a covariance matrix (similar scale of measurements). The first component (PC1) had a variance of 5.3728 (eigenvalue) and accounted for 93.3% of the total variance. The second component (PC2) had a variance of 0.3172 and accounted for 5.5% of total variability. The first two principal components together explained 98.8% of the total variability amongst the different sites (figure 2.11). The remaining components accounted for a very small proportion of variability and were not of great importance.

Table 2.9 ANOVA comparisons of intercept (a) and slope (b) of the allometric relationships between the sampling sites. *significant differences for p<0.05.

X – Y variables	**Intercept (a)**		**Slope (b)**	
	F	p	F	p
Length – Whorl	2.18	0.022*	2.08	0.030*
Length – Aperture	7.86	<0.001*	7.99	<0.001*
Length – Width	3.48	<0.001*	2.94	0.002*
Length – Depth	2.47	0.009*	2.36	0.013*
Length – Siphonal canal	6.67	<0.001*	6.27	<0.001*
Length – Thickness of lips	4.56	<0.001*	3.91	<0.001*

The multiplier of the variable shell length had the highest value for PC1, thus the first principal component mainly represents the effect of shell length on the variability between the sampling sites. Accordingly, PC2 represents the effect of shell shape on the variability because the aperture length, shell width and shell depth variables are the ones contributing the most on the explained variance between the sites. The distribution of sites along the x axis is stronger because PC1 explained 93.3% of the variability (figure 2.11). *Nassarius reticulatus* collected from Church Bay (wave-exposed) have the smallest shell length than all other populations (PC1 value closer to zero). Whelks collected from Malltraeth (semi-wave-exposed) and Trearddur Bay (sheltered) have the largest shell lengths (PC1 value further away from zero), with Aberffraw, Rhosneigr, Cymyran Bay, Carmel-Cemlyn, Rhoscolyn and Bull Bay in an intermediate position. *N. reticulatus* collected from Bull Bay and Rhoscolyn were the most aberrant (PC2 value furthest from zero) from the more elongated shell-form of whelks from Malltraeth and Aberffraw.

Figure 2.11 Principal component analysis for all the shell size variables of *Nassarius reticulatus* collected from 10 different sampling sites around the coast of Anglesey. Symbols:● sheltered site with moderately well sorted sediment,● sheltered site with moderately sorted sediment,● wave-exposed sites with moderately well sorted sediment, ● semi-wave-exposed sites with moderately well sorted sediment. Cemaes Bay (●) is a semi-wave-exposed site but no sediment sample was collected from there (sorting not available).



Based on the distribution of sites on the PCA plot (figure 2.11) grouping of the different sampling sites was attempted according to shore exposure and sediment variables (coloured symbols). The degree of sediment grain uniformity (sorting) was used as a sediment variable due to the fact that all sites presented here had the same type of fine sand sediments. Malltraeth and Aberffraw are semi-wave-exposed sites located on the west coast of Anglesey with moderately well sorted sediments, which are closely positioned on the PCA plot (yellow symbol). The Rhosneigr, Cymyran Bay and Carmel-Cemlyn sites are all sheltered areas with moderately sorted sediments and they also form another group of sites on the PCA plot (blue symbol). Bull Bay and Rhoscolyn, which are sheltered sites but with moderately well sorted sediments, are also closely grouped (green symbol). Church Bay (red symbol) is the only wave-exposed area and it is the most solitarily point on the PCA plot, thus the most dissimilar site, compared with the other sites.

2.3.7 Shell shape

The effect of shell shape in the Principal Component Analysis (figure 2.11) was described by the multiplier PC2, which had a higher value for the variables aperture length, shell width and depth. The shape parameter of PCA explained only 5.5% of the variability between sites. Ratios of shell variables such as aperture length to shell length (Crothers, 1985b; Lam & Calow, 1988; Bodon *et al.*, 1999) or shell length to shell width (Parsons, 1997b; Bodon *et al.*, 1999; Teusch *et al.*, 2002) have been extensively used for the description of shell shape in several gastropod species. During the present study the ratios of aperture length, shell width and shell depth with shell length were estimated and are summarised in table 2.10.

Table 2.10 Shell shape of *Nassarius reticulatus* described as mean (±SE) ratios of aperture length/shell length (AL/SL), shell width/shell length (SW/SL) and shell depth/shell length (SD/SL) for the 10 sampling sites. N: number of gastropods measured from each site.

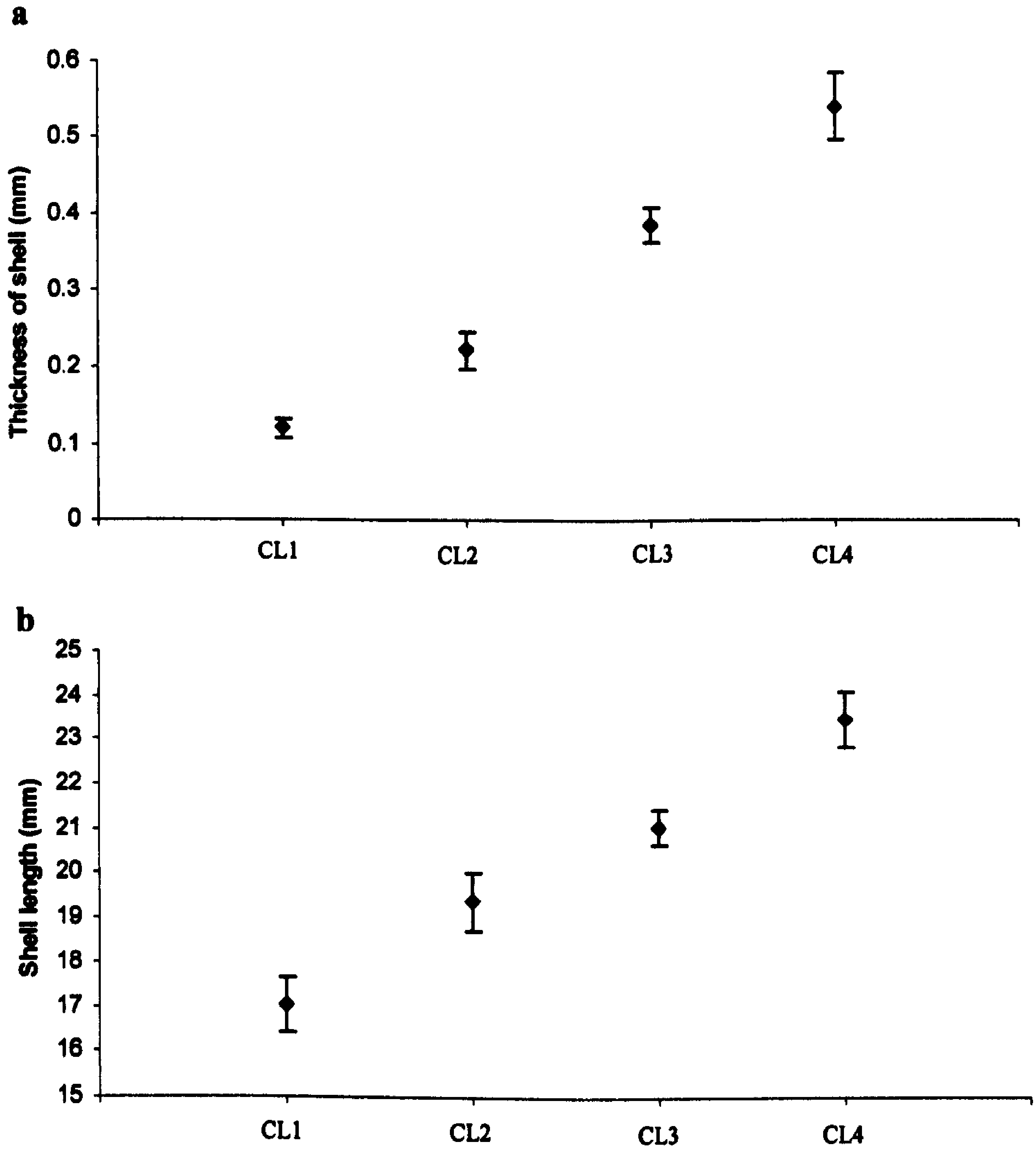
Sites	N	AL/SL	SW/SL	SD/SL
Malltraeth	63	0.51 ±0.003	0.47 ±0.003	0.43 ±0.002
Aberffraw	111	0.52 ±0.002	0.47 ±0.002	0.43 ±0.002
Rhosneigr	86	0.51 ±0.004	0.49 ±0.002	0.44 ±0.002
Cymyran Bay	108	0.51 ±0.004	0.49 ±0.002	0.45 ±0.003
Rhoscolyn	8	0.46 ±0.010	0.52 ±0.013	0.46 ±0.014
Trearddur Bay	15	0.48 ±0.007	0.49 ±0.008	0.45 ±0.004
Church Bay	46	0.51 ±0.005	0.49 ±0.003	0.44 ±0.002
Carmel-Cemlyn	39	0.51 ±0.004	0.50 ±0.004	0.45 ±0.004
Cemaes Bay	10	0.50 ±0.009	0.52 ±0.012	0.46 ±0.007
Bull Bay	13	0.49 ±0.008	0.52 ±0.007	0.48 ±0.007

Although a significant difference in shell shape between the sampling sites was found for all the ratios (AL/SL: F=6.57, p<0.001; SW/SL: F=21.18, p<0.001; SD/SL: F= 19.06, p<0.001) the variability could not be related to differences in shore wave-exposure. For example, no significant differences were found in the ratio AL/SL (F=0.91, p=0.490) between the wave-exposed site Church Bay, the sheltered sites Rhosneigr, Cymyran, Carmel-Cemlyn, Bull Bay and the semi-wave-exposed sites Malltraeth and Cemaes Bay. Similarly, no significant differences were found in the ratio SW/SL between the wave-exposed Church Bay site and the sheltered Cymyran, Rhosneigr and Trearddur Bay sites (F=0.45, p=0.719), or in the ratio SD/SL between the wave-exposed Church Bay, the sheltered Cymyran, Rhoscolyn, Trearddur, Carmel-Cemlyn and the semi-wave-exposed Cemaes Bay sites (F=1.54, p=0.178). *Nassarius reticulatus* from Aberffraw and Malltraeth had the lowest ratio for SW/SL and SD/SL indicating more elongate shells, whereas Bull Bay had the highest SW/SL and SD/SL ratio indicating wider and squat shells.

2.3.8 Condition of lips

The relationship between shell length and thickness of shell lips (figure 2.10f) indicated that as *Nassarius* increases in length the shell lip becomes more thickened. The condition of the shell lips (CL) can also be used to describe the progressive thickening of the shell margin and the formation of teeth on the inner shell lip. Animals at CL1 stage have thin sharp lips, animals at stage CL2 have thickened lips, while animals at stages CL3 and CL4 have thick lips with a row of single teeth and a row of elongated wide teeth respectively. Figure 2.12a shows the relationship between the condition of the lips and the thickness of the lips for *N. reticulatus* from all the sampling sites grouped together. A significant difference (F=84.58, p<0.001) was found in the lip thickness of whelks with the 4 different lip condition stages. Indeed, the shell lips become thicker as the condition of lips progresses from CL1 to CL4 stage. The relationship between lip condition and shell length (figure 2.12b) indicated a significant difference (F=49.73, p<0.001) between the shell length of gastropods of the 4 different lip condition stages. Gastropods with the CL4 stage have a greater shell length than those with anterior lip condition stages. As the whelks grow older and mature, shell lip thickness increases and a row of teeth (see figure 2.5) is formed on the inner lip, that gradually widens. The condition of the aperture lips has been frequently used as an indicator of age and maturity of gastropods. During the present study the shell lip condition was correlated with age and the results analysed and presented later in chapter 4.

Figure 2.12 a) Mean shell lip thickness (mm) of *Nassarius reticulatus* with lip condition stage from CL1 to CL4. b) Mean shell length (mm) of *Nassarius reticulatus* with lip condition stage from CL1 to CL4. Gastropods from all sampling sites are grouped together. The error bars represent the 95% confidence intervals (1.96· SE) of the means.



2.4 DISCUSSION

The survey of the distribution of *Nassarius reticulatus* around the Isle of Anglesey revealed well established populations along the west coast with less developed populations along the north coast. The highest abundance of *N. reticulatus* was observed at Malltraeth (23.4 whelks.trap^{-1}) and Aberffraw (20.6 whelks.trap^{-1}). Also a relatively high abundance was observed in the areas of Rhosneigr beach (13.6 whelks.trap^{-1}), Cymyran Bay (9.3 whelks.trap^{-1}) and Church Bay (4.4 whelks.trap^{-1}). Lower abundances were recorded at Newborough (0.4 whelks.trap^{-1}), Rhoscolyn (1.1 whelks.trap^{-1}) and Trearddur Bay (0.8 whelks.trap^{-1}). Also significant but relatively limited populations were observed along the north coast of Anglesey in the areas of Carmel Head (1.3 whelks.trap^{-1}), Cemlyn Bay (1.5 whelks.trap^{-1}), Cemaes Bay (0.4 whelks.trap^{-1}) and Bull Bay (1.3 whelks.trap^{-1}). By contrast not a single *N. reticulatus* was collected from the east coast of Anglesey, along the south-east facing Menai Strait and around the north-west areas of Holyhead harbour and the Inland Sea.

The index of dispersion was significantly higher than unity for all *Nassarius* populations around Anglesey, indicating a clumped distribution. Lambeck (1982) reported an aggregated distribution of *N. reticulatus* in the saline lake Grevelingen. According to Tallmark (1980) the dispersion of *N. reticulatus* in pure sand is less patchy, while in detritus rich sand the snails are more aggregated. The aggregated distribution of *N. reticulatus* is not a phenomenon of social behaviour. The gastropods are rapidly aggregated in areas where a carrion source is detected and immediately after feeding they are buried in the sediment in order to avoid predation. *N. reticulatus* living inside aggregations suffered less mortalities due to predation than individuals living outside aggregations (Stoner & Lally, 1994; Ray & Stoner, 1995).

The abundance of *Nassarius reticulatus* increased with depth, reaching maximum densities at 9-10m in Malltraeth, at 9-13m in Aberffraw and at 10-11m in Cymyran. The number of *N. reticulatus* collected per trap was very low in areas shallower than 4 m and deeper than 17m depth at all the sampling sites. *N. reticulatus* does not inhabit very shallow areas because the winter freezing temperatures can cause mortalities and eliminate the populations (Lambeck, 1982). During a survey of *N. reticulatus* in the saline lake Grevelingen (SW Netherlands) higher densities were recorded from stations located at 3m depth, whilst their densities in more shallow water (1m) and at deeper water (12 m) were lower than intermediate depths (Lambeck, 1984).

Although a wide range of shell sizes was present in all depths, a significant trend for smaller whelk sizes was noticed at a range of depths from 9 to 10m in Aberffraw, from 6 to 7m depth in Rhosneigr and from 7 to 8m in Malltraeth. At all sites larger shell sizes were found at the shallower and deeper depths. The predominant concentration of smaller shell sizes at water depths of 6-10 m could be explained by the presence of nursery grounds in the specific range of depths. Newly settled juvenile *Nassarius reticulatus* apparently spend their first 3 years in nursery grounds, which are suitable environments for optimum development and survival, are rich in food and protected from predators (Tallmark, 1980). When *N. reticulatus* mature they migrate to inshore waters where suitable substrates for egg laying are available, such as rocks and macroalgae (Lebour, 1937). According to Fretter & Shale (1973) maximum numbers of veligers of *N. reticulatus* were collected in Plymouth at 5 to 10 m depths, while densities were lower nearer the surface and deeper than 10m. The larvae sink rapidly when they are ready to metamorphose, thus density of juveniles and young adults may appear higher in depths of the same range (Fretter & Shale, 1973). However, Lambeck (1984) suggested that differences in sizes distribution of *N. reticulatus* at different depths in lake Grevelingen were the result of depth-dependent growth. In lake Grevelingen after one growing season animals at 2-3m depth had a mean shell length of 5-5.5 mm, animals at 5-6 m depth were bigger and had a maximum mean size of 6.8 mm, while animals at depths greater than 8m had the lowest mean shell length of 3.6 mm (Lambeck, 1984).

In order to identify in detail the optimum conditions for the development of *Nassarius reticulatus* populations, the locally variable environmental conditions (wave-exposure, sediment type, organic content and predation) were described for all sampling sites along the coast of Anglesey. Well developed populations were recorded in areas with different degrees of wave exposure. High numbers were collected from the wave-exposed shore of Church Bay, the semi-wave-exposed shores of Malltraeth and Aberffraw and the sheltered shores of Rhosneigr and Cymyran. Thus it appears that the scavenger *N. reticulatus* is well adapted to varying degrees of wave exposure.

High numbers of *Nassarius reticulatus* were found in moderately well sorted or moderately sorted sediments of fine sand (Malltraeth, Aberffraw, Rhosneigr, Cymyran and Church Bay). However, at Newborough, Trearddur, Carmel-Cemlyn and Bull Bay, where also the sediments were moderately well sorted fine sand, less developed populations were present. No whelks were found in very coarse sand (Holyhead), medium sand (Dulas Bay, Penmon Point and Tal-y-Foel), or very fine sand (Red Wharf Bay). In addition, areas with fine sand but poorly sorted sediments (Moelfre and Bangor Pier) were not inhabited by *Nassarius*. Similarly Lambeck (1984) indicated that higher densities of *N. reticulatus* were present in well sorted fine sand sediments and relatively low densities were recorded in areas with medium sand.

The organic content and silt content in the sediment was not particularly high (organic <0.5%, silt <2%) in the west and north areas of Anglesey, where the abundance of *Nassarius reticulatus* was greater. The sediment collected from Holyhead harbour and Cemlyn Bay had a higher organic content of (2.06 and 1.77 % respectively) and a higher silt content (2.99 % and 4.79 % respectively). However, no gastropods were collected from Holyhead and only a few were present at Cemlyn Bay. There were no *Nassarius* present along the east and south coast of Anglesey where sediments were generally richer in organic and silt content. Lambeck (1984) recorded a very low density of *N. reticulatus* in sediments with an organic content >3.9%. Although most of the sampling sites along the west coast were characterised as sheltered, the overall area is highly affected by the prevailing SW winds. Increased wave action causes re-suspension of sediments, and as a result nutrients and organic material are not deposited in the sediment. According to Dorgelo & Leonards (2001) formation of offshore under-currents along the NW shore of lake Maarsseveen (Netherlands), which is affected by the predominantly westerly winds, reduces the particle deposition and detritus content in the sediment. Similarly, in the present study the organic content of the sediment was lower in areas with increased water circulation, such as the west coast of Anglesey, while sediment samples collected from the less wave-exposed east coast were richer in organic material. Hawkins *et al.* (1992) reported that primary production is about 6 times lower along wave-exposed rocky shores compared with primary productivity in more sheltered shores. Therefore, it is not surprising that the sediments adjacent to wave-exposed rocky shores had a lower organic content than sediments of sheltered sites. A high level of organic waste discharge in Holyhead harbour might be responsible for the higher percentage organic content of the sediment. Waste dumping in seawater has a direct effect on the benthic fauna which is exposed to the sedimentation of particulate matter rich in nutrients and bacteria (Clark *et al.*, 1999). Also the higher organic content in the area of Cemlyn Bay could be the result of increased productivity caused by thermal pollution from the adjacent Wylfa nuclear power plant.

In order to describe the sediment conditions that are more favourable for the development of *Nassarius* populations, the mean abundance of gastropods (all sites grouped) was correlated with mean grain size, silt content and organic content (table 2.6). A high abundance of *N. reticulatus* was found in sediments where the silt content ranged between 0.2 and 3%, the organic content ranged from 0.4 to 0.5 % and the mean grain size ranged from 2.78 to 2.89 phi units (fine sand). Very few whelks were collected from sediments with low (<0.19%) or high (>3%) silt content and with low (<0.35%) or high (>0.6%) organic content. Lambeck (1982) observed that the number of *N. reticulatus* in lake Grevelingen (SW Netherlands) decreased as the median grain size, the organic content and the silt content increased. The greatest density of *Nassarius* (gastropods.m^{-2}) in lake Grevelingen was found in sediments with 1-5% silt content and with 0-0.5% organic content. Populations of *N. reticulatus* become established in fine sand and well-oxygenated sediments that are not particularly rich in organic content. Sediments rich in silt and organic matter have lower oxygen and higher sulphide concentration due to increased bacterial activity (Raffaelli & Hawkins, 1999) and often develop anaerobic conditions. The more sensitive benthic species can be eliminated due to the reduction of oxygen concentration because of enhanced bacterial activity, and only opportunistic species such as the polychaete *Capitella capitata* benefit from organic enrichment (Clark *et al.*, 1999). Although *Nassarius* can tolerate short periods of oxygen deficiency (Rosenberg, 1977), activity and growth may be adversely affected by a temporarily lowered oxygen concentration (Bannink *et al.*, 1984).

The highest abundance of predatory crab species was observed along the Menai Strait (3.7-7 predators·trap^{-1}) and in the Inland Sea (6.8 predators·trap^{-1}), where no *Nassarius reticulatus* were present. The number of predators caught in the traps was low along the west Anglesey coast where the abundance of *N. reticulatus* was higher (0-0.8 predators·trap^{-1}). The sampling stations along the north coast of Anglesey, where smaller numbers of *Nassarius* were found, were also characterised by low abundance of predators (0.1-1.1 predators·trap^{-1}), and only in Cemaes Bay a slightly increased number of predators per trap was observed (2.4 predators·trap^{-1}).

Overall the survey of *Nassarius reticulatus* along the Anglesey coast reveals well developed populations on the west coast of the island, which is most affected by the local south-west prevailing winds. It is likely that a greater amount of carrion is transferred into the shallow sheltered waters along the west coast due to increased wind action, and this source of food can be effectively used by the scavengers that congregate there (see also Tallmark, 1980; Lambeck, 1982). In addition, west coastal areas of Anglesey are characterised by moderately well sorted and fine sand sediments that are well oxygenated and have a low organic and silt content. The very low number of predators that was recorded along the west coast of Anglesey may also be an important factor for successful juvenile *Nassarius* development.

Physical barriers may explain the limited or completely absent populations of *Nassarius reticulatus* in areas that are also located along the west coast of the island. The sites of Newborough and Inland Sea are characterised by the formation of extensive sand banks, which do not offer a suitable rocky environment for egg laying by the adult female whelks. In addition these areas are characterised by increased numbers of predatory crabs. Tallmark (1980) suggested that the lower abundance of *N. reticulatus* in the southern part of Kvarnbukten bay (Sweden) was because the area was isolated by a sand barrier which prevented immigration. The beaches at Newborough and the Inland Sea sites are affected by strong drifting conditions due to the strong tidal currents that occur in these areas. Tallmark (1980) observed that extreme tidal currents may help to explain the lower numbers of *N. reticulatus* in Kvarnbukten bay in stations that were periodically drained at low tide. When recruitment levels of *N. pauperatus* were compared between two sandflats in Gulf St.Vincent in South Australia, a higher density of recruits was found on sandflats where there were extensive pools of water exposed during low tide (McKillup *et al.*, 1993). Finally, the wave-exposed and rocky coastline of west Holy Island located between Trearddur Bay and Holyhead harbour probably represents a great constraint regarding larval dispersion and settlement.

Smaller populations of *Nassarius reticulatus* were also present along the north coast of Anglesey. The residual water current, which flows through the Irish Sea and is directed from the west towards the north Anglesey coast, may be responsible for transferring a significant number of *Nassarius* larvae from the well established west populations to the north coast. The north coast is occasionally affected by north-west or north-east winds that could enrich the shallow waters with carrion and thus maintain these populations. In addition, successful establishment of a *Nassarius* population in these areas is facilitated by the well oxygenated and moderately sorted fine sand sediments, which characterise most sites along the north coast. A small *Nassarius* population was only found in the Cemaes area where numerous predators were observed.

The complete absence of *Nassarius reticulatus* from the east and south coasts of Anglesey could be attributed to the non optimum sediment type (medium sand, very fine sand or poorly sorted fine sand), and to the higher organic and silt content of sediments that enhance the development of anaerobic conditions. The east and south coast are very rarely affected by wind, so circulation of water and oxygenation of sediments may not be promoted. In addition, the powerful tides in these areas are responsible for high turbidity in the water and increased amounts of silt in suspension. Finally, the very high abundance of predatory crabs that was observed in the Menai Strait is likely to inhibit whelk growth and survival.

Comparison of the morphological shell parameters between the different sites revealed significant similarities amongst all the sheltered and most of the semi-wave-exposed habitats. Whelks collected from the wave-exposed site of Church Bay were significantly smaller regarding all the parameters measured and had thinner shell lips than *Nassarius* from all the other sites. *Nassarius* collected from the semi-wave-exposed Malltraeth site had significantly bigger shells compared to the other sites. The shell length of whelks collected from the sheltered Trearddur Bay was also higher, although not significantly different from the rest of the sheltered sites. Individuals collected from sheltered sites such as Rhoscolyn and Trearddur Bay had the thickest shell lips. Significant differences in the allometric relationships between the various shell dimensions from the different sampling sites underlined the variability of shell growth under different environmental conditions.

The relationships between shell length and whorl length, aperture length, shell width and shell depth were negatively allometric in *Nassarius reticulatus* collected from most sites, indicating that whorl and aperture length increase more slowly than shell length. Negatively allometric relationships between shell length and aperture length are quite common in many gastropods in which shell length growth ceases at maturity (Vermeij, 1980). In some of the sites the above relationships were isometric, indicating that the dimensions of the shell grow at the same rate. In the earlier ontogenetic stages the shape of the gastropod shell changes in proportion with shell length, thus the relationship of shell length with other shell parameters appears to be isometric (Vermeij, 1980). Allometry and eventually doming (increase in relative spire height) appears in larger gastropods (Vermeij, 1980).

The relationship between shell length and lip thickness was positively allometric in *Nassarius reticulatus* collected from most sampling sites, indicating that thickening of shell lips is faster than the increase in shell length, although in some sites the relationship was isometric. Spight & Lyons (1974) observed that the snail *Ceratostoma foliatum* alternates a spiral growth phase, where an increment of shell is added to the growing edge, with a radial growth phase, where shell material is laid down in the form of a varix or thickening of the outer lip. When the radial phase begins to predominate over the spiral phase, the formation of varices or the thickening of lips increases disproportionally with shell length, resulting in a positively allometric relationship between shell length and lip thickness (Spight & Lyons, 1974).

Grouping of sites on the PCA plot indicated similarities regarding shell length and shape between *Nassarius reticulatus* collected from sites with similar sediment and shore exposure conditions. *Nassarius* collected from Malltraeth and Aberffraw, which are both semi-wave-exposed sites located on the west coast of Anglesey with moderately well sorted sandy sediments, have similar shell morphologies. Also whelks from sheltered areas with moderately sorted sandy sediments, such as Rhosneigr, Cymyran Bay and Carmel-Cemlyn, display similar shell development. Whelks from Bull Bay and Rhoscolyn which are sheltered sites but with moderately well sorted sediments, were also closely grouped. Finally, *Nassarius* collected from the wave-exposed Church Bay had very different shell morphologies than gastropods from all the other sites examined in the present study.

PCA analysis indicated that shell length could explain 93.3% of the variability in *Nassarius reticulatus* morphology amongst the different sites and shell shape explained a further 5.5% of the observed variability, and confirmed the initial differences observed between sites during comparison of means. Individuals collected from the wave-exposed site Church Bay, had by far the smallest shell lengths than whelks in all the other populations. Gastropods collected from the semi-wave-exposed Malltraeth and the sheltered Trearddur Bay had the larger shell lengths, although the remaining sheltered and semi-wave-exposed sites were not very different. Likewise, Parsons (1997a) reported that adults of *Bembicium vittatum* from sheltered sites had longer shells than adults from wave-exposed shores. The formation of larger shells in sheltered environments offers effective protection against predation. Predation is higher along sheltered shores in comparison with wave-exposed shores (Ekaratne & Crisp, 1983; Richardson & Brown, 1990). The relative shell strength increases roughly with the 1/3 power of shell length, thus gastropod shells of greater length are stronger than the ones of smaller length (Tokeshi *et al.*, 2000). Large shells require more energy expense and increase the probability of claw damage by the predator, whereas small shells are weaker and require exponentially less force to achieve breakage (Juanes, 1992). Furthermore, the development of an elongated shell is probably the most efficient way in which a gastropod can increase its body size and at the same time restrict aperture width (Seed, 1978). The longer shells of *Nucella lapillus* may discourage predation by gulls and at the same time, due to the smaller apertures, predation by crabs is avoided (Crothers, 1973).

Although shell lip thickness was not a significant factor in the PCA analysis, the comparison of means indicated that the shell lips of the wave-exposed Church Bay *Nassarius* were thinner. By contrast, *Nassarius reticulatus* collected from the sheltered sites of Rhoscolyn and Trearddur Bay had significantly thicker shell lips. Several studies in the literature report that thicker shell lips offer a great advantage in gastropods inhabiting sheltered environments because they are less susceptible to predation. For example, *Nucella lapillus* from sheltered shores are characterised by thicker shells and smaller apertures that could inhibit predation by *Carcinus maenas* (Gibson, 1970; Seed, 1978; Hughes & Elner, 1979). The narrower aperture of the sheltered shore gastropods excludes the chelae of attacking crabs and the thicker more robust shell is harder to break (Hughes & Elner, 1979). On the contrary, *N. lapillus* populations from wave-exposed shores had shorter and wider shells, with larger apertures and larger foot areas (Seed, 1978). The larger foot area significantly increases the power of adhesion, which is clearly an advantage in habitats experiencing considerable wave action (Seed, 1978; Hughes & Elner, 1979; Smith, 1981b). Similarly, *Littorina* species found on sheltered shores have larger and thicker shell with a narrower aperture in order to prevent crab attacks, while conspecifics from wave-exposed shores have smaller and thinner shells with a large foot and aperture in order to prevent dislodgement by wave action (Johannesson, 2003).

Overall these findings indicate that the small shell size, wide aperture and large foot of gastropod species from wave-exposed shores offer greater resistance to dislodgment by waves, whereas the large shell size, thicker shell and small aperture of individuals from protected shores are adaptations in resisting predation by shore crabs. During the present study it was confirmed that *Nassarius reticulatus* from sheltered shores had larger and thicker shells as means of protection against crab predation. However, aperture size was not larger in *Nassarius* from wave-exposed shores. All the examples of wider apertures in wave-exposed-shore gastropods that were presented above, refer to species living attached to rock surfaces. *N. reticulatus* is a benthic species that spends most of its time buried within the sediment, thus dislodgment by wave action may not be a constraint for living in exposed situations.

The negative allometric relationships between shell length and both shell width and shell depth of *Nassarius reticulatus* from Malltraeth, Aberffraw, Rhosneigr and Carmel-Cemlyn indicated that shell length increased faster than shell width and depth resulting in a more elongate shell shape. Additionally, the PCA results regarding shell shape indicated that *N. reticulatus* collected from Aberffraw and Malltraeth had more elongated shell forms, although only 5.5% of the inter-site variability could be explained by the shape factor. Further comparisons of shape, described as ratios of aperture length, shell width and shell depth with shell length, also indicated that Aberffraw and Malltraeth had more elongate shells. However, no significant differences were found regarding shell shape between the sheltered, semi-wave-exposed and wave-exposed sites.

Shell polymorphism is an adaptive mechanism that promotes survival and optimum growth in habitats with different environmental constraints, and it appears to be more common in intertidal gastropod species that lack a pelagic larval dispersion phase (Johannesson *et al.*, 1997). Shape variations in *Bembicium vittatum*, which develops directly from benthic eggs, are genetically determined, whereas in *Austrocochlea* *constricta*, which has a short mobile planktonic phase, shape variations are the result of physiological flexibility (phenotypic plasticity) (Parsons, 1997b). Species with pelagic larvae are able to recruit into various habitats, and as a result strong local adaptation and formation of distinct ecotypes in contrasting habitats is prevented (Johannesson, 2003). However, plasticity of shape in species with greater dispersal abilities can be more pronounced at locations where the gene flow is wider, thus resulting in phenotypic variability between populations in different areas as an adaptation to environmental conditions (Parsons, 1997b). Janson (1987) reported that populations of *Littorina littorea* (planktonic larvae) have similar shell shapes in wave-exposed and sheltered environments, while populations of *L. saxatilis* (direct development) differ significantly in shell shape between wave-exposed and sheltered shores. Accordingly, in this study populations of *Nassarius reticulatus*, which are characterised by a long pelagic larval phase, had similar shell shapes in sheltered, semi-wave-exposed and wave-exposed shores around Anglesey.

Several aspects regarding the ecology of *Nassarius reticulatus* were studied in this chapter. The distribution of *N. reticulatus* around the Isle of Anglesey revealed well established populations on the west coast, smaller populations mainly on the north coast and a complete absence of gastropods from the east and south coast of the island. The abundance of *Nassarius* was maximal in depths of 9 to 13 m. The gastropods inhabited a range of wave-exposed to sheltered environments with relatively low predation pressure. Moderately well sorted, sandy sediments with a relatively low percentage of silt and organic content were mostly preferred by the gastropods. The shell morphology of *N. reticulatus* was significantly different between sites with variable degrees of wave-exposure and predation pressure. Gastropods from sheltered shores developed a larger and thicker shell as an adaptation to increased predation by crabs in such environments. However, the shape of gastropods was similar between sheltered, semi-wave-exposed and wave-exposed environments, which is a common feature in gastropods with long pelagic larval phases.

CHAPTER 3

Population dynamics and growth of *Nassarius reticulatus*

3.1 INTRODUCTION

Few studies have investigated the population dynamics and growth patterns of *Nassarius reticulatus*. Tallmark (1980) studied the population dynamics of *N. reticulatus* in Kvarnbukten Bay, Gullmar Fjord (Sweden). Recruitment, growth and migration patterns were described using length-frequency distribution analysis and the growth of individuals was estimated from growth rings developed on the external shell surface and from the thickness of the shell lips (Tallmark, 1980). However, the negligible shell growth of many large snails prevented their age determination from shell rings (Tallmark, 1980). Based on the number of shell rings Tallmark (1980) suggested that the maximum age attained by *N. reticulatus* was at least 15 years, while Bryan *et al.* (1993) reported a maximum of 17 annual checks from a population in SW England. Lambeck (1984) studied the population dynamics of *N. reticulatus* in the recently colonised lake Grevelingen (SW Netherlands), where conditions of growth were optimal due to the low density of the population and the high, almost *ad libitum*, availability of food. Age classes were distinguished according to changes in modal size classes in the size-frequency distributions (Lambeck, 1984). Barroso *et al.* (2005a) estimated the age and growth of *N. reticulatus* in the Ria de Aveiro (Portugal) from length-frequency analysis of the population distributions and from external and internal growth rings of the shell.

The population dynamics of other nassariid species have been investigated in several areas. Morton & Chan (2004) demonstrated that *Nassarius festivus* in Hong Kong has a short life span ranging from 22 to 29 months and a high growth constant (K=2-2.7). McKillup & McKillup (1997) found that *N. dorsatus* at Coorooman Creek (Central Queensland, Australia) recruited throughout the year, grew rapidly, had an extremely short life span and died just 2 months after settlement. Crichton (1942) suggested that *Bullia vittata* was able to attain its full size within a period of < 6 months, while Thilaga *et al.* (1987) reported that the same species in Porto Novo, India, attained shell heights of 17, 34.5, 42.5 and 50.2 mm during the first 4 years of life respectively. Brown (1971) reported that the life span of *B. digitalis* was at least 5 years. The longest life cycle of 40 years in a nassariid has been described by Curtis (1995) regarding a population of *Ilyanassa obsoleta* at Cape Henlopen sand-flat (Delaware Bay, USA). Morton & Chan (2004) suggested that *N. festivus* has a shorter life span and higher growth rate than the nassariids *I. obsoleta* and *N. reticulatus* because it is a sub-tropical species. Even regarding the same species, populations from different localities can have different life spans or growth patterns, due to variations in temperature, food availability and shore topography (Morton & Chan, 2004).

The demographic characteristics of a population, such as abundance, age structure, birth rates, age specific growth rates and mortality, reflect the ecology of a species and can provide information about the spatial and temporal extent of environmental stresses (Cerrato, 1980). There are several methods for estimating the demographic parameters of a population. Population attributes can be deduced from size-frequency histograms, where recognition of peaks can be used for determination of recruitment, growth and age structure of populations (Cerrato, 1980). Methods for estimating growth by length-frequency analysis are based on the aspect that each modal class corresponds to a cohort and represent different age classes determined at regular intervals (Morales-Nin, 1992). The presence of modes in length-frequency distributions depends on the distance between the medians, the extent of the variance, the proportion of each age class in the population and the size of the sample studied (McDonald & Pitcher, 1979). Methods for the determination of demographic parameters from the population size-structure assume that recruitment in the population takes place in discrete pulses, resulting in annual non-overlapping cohorts that can easily be distinguished in subsequent years (Grant *et al.*, 1987).

However, in several cases modes within a size-frequency distribution are not always easily identified (Cerrato, 1980). The number of clearly defined modes can be reduced by poor recruitment in some years, variation in the duration of the breeding season, mortality, or if sampling takes place at a time long after the most recent spawning event (Cerrato, 1980; Grant *et al.*, 1987). Older age groups usually tend to merge together because the variation in size between individuals increases with age, while growth rates decline (Cerrato, 1980; Grant *et al.*, 1987). Consequently, such methods can only be applied to populations with seasonal recruitment, where the identity of annual classes is maintained and where growth is sufficiently fast in order to avoid super-imposition of successive age classes (Morales-Nin, 1992).

Even in cases of overlapping cohorts, useful information about the population can be obtained from a time sequence of length-frequency distributions by using methods that are able to identify the composition of the underlying cohorts (Grant *et al.*, 1987). In most cases the sizes of individuals of the same age class approximate a normal distribution, thus a mixture of normal distributions in a population can be dissected into its components by using either graphical methods (e.g. Bhattacharya's method, 1967) that determine the area of cumulative frequency, or computer methods based on a maximum likelihood statistical analysis (Grant *et al.*, 1987; Morales-Nin, 1992). After determining the mean size of each component, the growth progress of each cohort in time can be followed and a von Bertalanffy growth curve can be fitted to the data. Such methods for length-frequency analysis have been used for describing the growth of *Nassarius reticulatus* in the Ria de Aveiro (Portugal) (Barroso *et al.*, 2005a), *N. festivus* in Hong Kong (Morton & Chan, 2004) and *Bullia vittata* in Porto Novo (India) (Thilaga *et al.*, 1987).

The von Bertalanffy growth model assumes that the growth increment is a linear function of the initial size with a negative slope (Hughes, 1980). This assumption is based on two further assumptions, that the assimilation rate is proportional to the square of a standard linear dimension of the body and that the metabolic rate is proportional to the cube of the same dimension (Hughes, 1980). For example, Hughes (1980) reported that departures from either of these assumptions in *Littorina nigrolineata* resulted in a non linear function between growth increment and initial size, thus the von Bertalanffy model predicted an overestimated shell size. The von Bertalanffy growth curve has been successfully used to describe the growth of nassariid species such as *Bullia rhodostoma* (McLachlan & Van-der Horst, 1979) and *Nassarius festivus* (Morton & Chan, 2004).

Grant *et al.* (1987) have discussed the usefulness of size frequency data for determining demographic parameters using graphical or computer methods. They concluded that size frequencies are only useful when the age classes are well separated and the size of sample very large (~1000 animals). It is very difficult or impossible to determine the number of age classes and the longevity of a population using any method of size-frequency histograms analysis, especially when the older size classes are poorly represented (Grant *et al.*, 1987). Ideally, the conclusions of a size-frequency analysis have to be validated by comparison with growth data derived from tagging or marking experiments (Grant *et al.*, 1987). Marking-recapture experiments are often used for the estimation of abundance, growth, survival and mortality rates in gastropod species. Several different techniques of marking have been used, such as fluorescent markers, coloured dyes or physical marks on shells (drilling, numbered tags) (Grant *et al.*, 1987). When the initial size and the size at recapture are available, and the time between marking and recapture is known, then growth can be determined by using the methods of Ford-Waldford (Ford, 1933), Gulland & Holt (1959) and Munro (1982). Also the size of a population, survival and mortality rates can be estimated from mark-recapture data using Jackson's (1939) positive and negative methods, the Fisher-Ford's method (1947), the Jolly's stochastic method (1965) and the Manly-Parr method (1968).

Tallmark (1980) estimated the growth of *Nassarius reticulatus* by marking 3800 individuals, from which 40% were recaptured at least once and many were recaptured several times. Marking of *N. reticulatus* with tags did not retard the growth rate of snails bigger than 15 mm (Tallmark, 1980). Hughes (1980) used the distinctive growth checks that were developed on the shell of *Littorina nigrolineata* during handling in order to obtain growth increment data. The von Bertalanffy growth curve that was fitted to the mark-recapture data predicted a faster growth rate than that estimated using length-frequency analysis, however, the results of the two methods were still similar and both acceptable (Hughes, 1980). Mark and recapture experiments have commonly been employed for the determination of growth patterns in several gastropod species, such as *Nassarius dorsatus* (McKillup & McKillup, 1997), *Ilyanassa obsoleta* (Curtis, 1995), *Helisoma anceps* (Fernandez & Esch, 1991), *Strombus gigas* (Appeldoorn, 1988), *Littorina littorea* (Moore, 1937), *L. neritoides* (Roberts & Hughes, 1980), *Cerithium nodulosum* (Yamaguchi, 1977), *Japeuthria ferrea* and *J. cingulata* (Ota & Tokeshi, 2000), *Haliotis rubra* (Prince *et al.*, 1988b), *Purpura lapillus* (Moore, 1938) and *Concholepas concholepas* (Rabi & Maravi, 1991).

Growth in gastropods has usually been determined through size-frequency distribution studies and individual mark-recapture techniques (Ekaratne & Crisp, 1984). Growth can also be studied directly in organisms raised in captivity, where length progression can be followed throughout the rearing period, although it has to be noted that growth under laboratory conditions is not always equivalent to growth in the natural environment (Morales-Nin, 1992). The most commonly employed measure of growth for coiled gastropods is the change in shell length, measured from the apex to the furthermost point of the body whorl along the axis of coiling (Ekaratne & Crisp, 1984). Linear increments in shell size are the results of new material being secreted by the mantle around the lip of the shell (Hughes, 1986). In simple helical growth the internal architecture of the shell remains unaltered, except for a general thickening of the wall by secretions of the inner mantle (Hughes, 1986).

The amount of energy available for growth is determined by the ingestion and the respiration rate of an organism, which are functions of temperature, food supply and body size (Hughes, 1986). As a result growth variations are likely to be dependent on both temperature and body size. Tallmark (1980) indicated that temperature was one of the factors affecting locomotory activity, migration, spawning and growth of *Nassarius reticulatus*. Temperature fluctuations affect food quality and quantity, feeding activity of organisms and consequently growth (Ekaratne & Crisp, 1984; Moran *et al.*, 1984). Higher temperatures have been correlated with an increase in growth rate of *N. reticulatus* (Lambeck, 1984; Barroso *et al.*, 2005a), *N. obsoletus* (Scheltema, 1964) and *N. festivus* (Morton & Chan, 2004). Shell growth rate increases when the temperature is higher during the summer months in several gastropod species, such as *Ilyanassa obsoleta* (Curtis, 1995), *Helisoma anceps* (Fernandez & Esch, 1991), *Littorina nigrolineata* (Hughes, 1980), *Polinices duplicatus* (Edwards & Huebner, 1977), *Nucella lapillus* and *L. littorea* (Ekaratne & Crisp, 1984), *Morula marginalba* (Moran *et al.*, 1984), *Monodonta labio* (Takada, 1995), *Japeuthria ferrea* and *J. cingulata* (Ota & Tokeshi, 2000).

Following sexual maturity less energy is devoted to somatic growth because more energy is allocated to reproduction, thus growth rate declines with age (Hughes, 1986). Tallmark (1980) and Barroso *et al.* (2005a) demonstrated that growth of *Nassarius reticulatus* was faster during the first years of life and when the whelks became sexually mature, growth rate decreased and became irregular. Similar observations have been widely reported for other nassariids including *N. festivus* (Morton & Chan, 2004), *Bullia vittata* (Thilaga *et al.*, 1987) and *Ilyanassa obsoleta* (Curtis, 1995). Growth rate is size dependent and declines after sexual maturity in many gastropods, such as *Helisoma anceps* (Fernandez & Esch, 1991), *H. rubra* (Prince *et al.*, 1988b), *Littorina nigrolineata* (Hughes, 1980), *Polinices duplicatus* (Edwards & Huebner, 1977), *Morula* *marginalba* (Moran *et al.*, 1984), *Cerithium nodulosum* (Yamaguchi, 1977), *Concholepas concholepas* (Rabi & Maravi, 1991), *Monodonta labio* (Takada, 1995), *Japeuthria ferrea* and *J. cingulata* (Ota & Tokeshi, 2000). The net growth efficiency of *Polinices alderi* declines with increasing body size and is generally lower when the snail enters its reproductive phase because less absorbed energy is available for growth (Ansell, 1982).

When somatic growth coincides with gonad development or breeding, the reproductive claim on absorbed energy depresses somatic growth causing seasonal changes in growth efficiency (Moran *et al.*, 1984; Hughes, 1986). According to Ekaratne & Crisp (1984) the gonad development of *Patella vulgata* is responsible for depression of shell growth during the summer months. However, in cases where gonad development coincides with months of low temperature and low growth, depression in growth rate will be probably masked (Ekaratne & Crisp, 1984). For example, growth rates in *Nucella lapillus* and *Littorina littorea*, where gonad development takes place during winter and spring, do not become reduced during the summer months (Ekaratne & Crisp, 1984). Similarly, the breeding period of *Japeuthria ferrea* and *J. cingulata* in Amakusa (SW Japan) (Ota & Tokeshi, 2000) and of *Nassarius festivus* in Hong Kong (Morton & Chan, 2004) occurs during the winter months and is synchronised with the period of no growth. Cessation of shell growth at the stage of sexual maturity is commonly accompanied by a pronounced thickening of the apertural margin, often with tooth-like sculpturing of the inner rim (Hughes, 1986). These features increase the strength of the shell and may defend the aperture against intrusion by predators (Hughes, 1986). Previously thickened margins become ridges as new, thinner shell is extended beyond them in episodes of growth (Hughes, 1986).

The rate of secretion of shell material is reduced during starvation, thus limited food supply retards growth of individuals in many natural populations of gastropods (Hughes, 1986). McKillup & Buttler (1983) indicated that the adult size of *Nassarius pauperatus* varied significantly according to food availability and that there was a significant correlation between hunger level and mean shell size in the field. McKillup & McKillup (1997) demonstrated that the growth rate of *N. dorsatus* was affected by the food availability in the field. Starvation in *N. festivus* increased the frequency of feeding, the proportion of individuals engaged in feeding and the time spent on feeding, while it decreased the time needed for reaching a food source and overrode the risk of predation (Morton, 1990; Morton *et al.*, 1995). During periods of reduced food availability, activity (locomotion, reproduction, growth) and therefore oxygen consumption may be decreased in order to conserve energy (Bayne & Newell, 1983). However, scavengers do not decrease their standard (resting) metabolism when they are starving, so that they are able to come out of the sand immediately when food becomes available (Cheung & Wong, 2001).

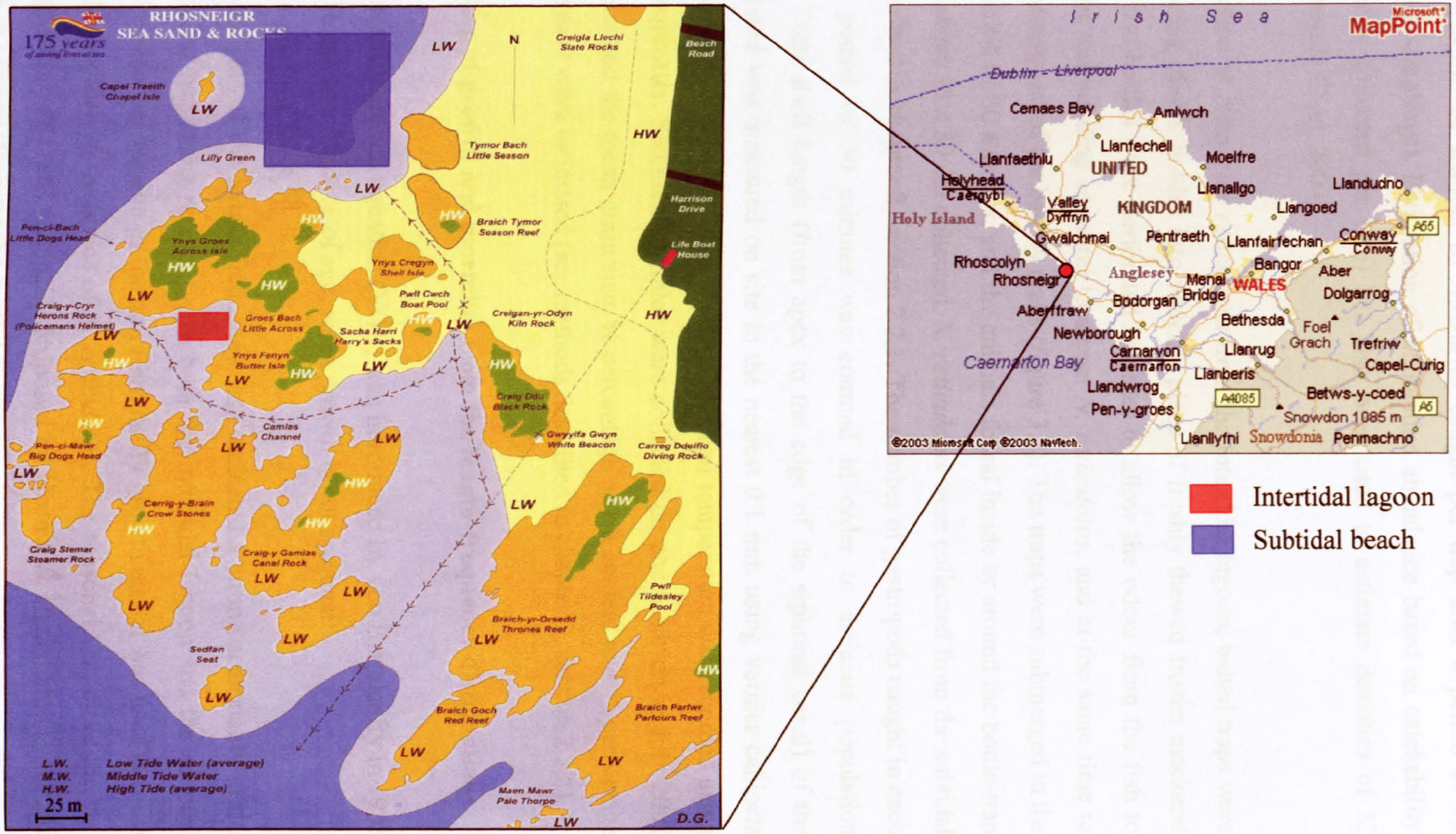
The aims of the present study were: 1) to estimate the seasonal variation in catchability of *Nassarius reticulatus* at Rhosneigr, Anglesey 2) to evaluate the differences in *N. reticulatus* catchability between an intertidal lagoon and a subtidal beach at Rhosneigr, 3) to describe the recruitment patterns of *N. reticulatus*, 4) to estimate the mortality of *N. reticulatus*, 5) to determine the growth patterns of *N. reticulatus* in the two habitats from length-frequency distributions using the Modal Progression Analysis (graphical method) and the LFDA analysis (computer method), 6) to compare estimates of shell growth from these two methods with measurements of growth increments from marked and recaptured *N. reticulatus* in the intertidal lagoon, 7) to evaluate in the laboratory seasonal changes in shell growth, the growth of different size classes, gender differences in growth, and differences in growth of whelks of different origin (intertidal lagoon, subtidal beach) and 8) to determine the effect of limited food availability on survival and growth under laboratory conditions.

3.2 MATERIALS AND METHODS

3.2.1 Sampling

The distribution of *Nassarius reticulatus* around the Isle of Anglesey was described in detail in chapter 2.3.1. The area of Rhosneigr located along the west coast supports a well-developed population and was selected for a regular monthly sampling survey in order to study the population dynamics of *N. reticulatus*. Seasonal variations in catchability and size distributions were investigated monthly between April 2002 and December 2003. Two different habitats were investigated: a rocky-enclosed intertidal lagoon (~273 m^2) and a subtidal sandy beach (~3906 m^2) (figure 3.1). Due to the clumped distribution of *N. reticulatus* (Chapter 2, section 2.3.2) density of whelks per

Figure 3.1 Rhosneigr (Anglesey, North Wales). Location of the intertidal lagoon and subtidal beach sampling sites surveyed monthly from April 2002 to December 2003



unit area could not be accurately estimated at each site. Instead population abundance was evaluated from the number of *Nassarius* caught per trap after a period of 30 minutes (timed samplings). Estimation of population abundance based on catchability data (gastropods collected per trap) has also been used to estimate densities of *N. festivus* (Morton & Chan, 2004).

For the collection of *Nassarius reticulatus* from the intertidal lagoon, baited traps were constructed using plastic bottles into which a piece of freshly thawed frozen mackerel was placed. The bottles were perforated in order to allow the odour from the fish to escape into the surrounding seawater to attract *N. reticulatus*, and at the same time to avoid the consumption of the bait by crabs (figure 3.2). The traps were submerged in the water for a period of 30 minutes, and the animals found inside or around the bottle-trap in an area of about 50 x 50 cm collected. *N. reticulatus* were collected from the subtidal beach as described in chapter 2, section 2.2.1. The number of gastropods caught in each trap after a period of 30 minutes was counted in order to estimate population catchability. Total shell length (from apex to the edge of the siphonal canal) of the animals collected was measured on site to the nearest 0.1 mm using vernier callipers and the animals returned to the water. Surface seawater temperature was recorded using a digital thermometer. Sediment samples were collected on one occasion in July 2002 from both areas and the mean grain size, % percentage of silt content and % percentage of organic content were estimated (see methods described in chapter 2, section 2.2.5).

3.2.2 Modelling of growth and mortality from the length-frequency distributions

The von Bertalanffy growth equation (VGB) was introduced by von Bertalanffy in 1938 and predicts length as a function of age, when growth is not seasonal:

$$L(t) = L_\infty \left[1 - \exp\left[-K\left(t - t_0\right)\right]\right]$$

Preliminary analysis of the length-frequency data indicated a strong seasonality in the growth pattern of *Nassarius reticulatus* in both the intertidal lagoon and the subtidal beach, thus the seasonal version of the von Bertalanffy growth model was used in order to describe the population dynamics of *N. reticulatus*. The seasonal version of the von Bertalanffy growth curve was originally proposed by Hoenig & Hanumara (1982) and relates length (L) to age (t) using the formula:

$$L(t) = L_\infty \left[1 - \exp\left[-K\left(t - t_0\right) + \left(C \cdot \frac{K}{2\pi}\right)\sin 2\pi\left(t - t_S\right) - \left(C \cdot \frac{K}{2\pi}\right)\sin 2\pi\left(t_0 - t_S\right)\right]\right]$$

Figure 3.2 Sampling of *Nassarius reticulatus* in the intertidal lagoon using baited plastic bottles. The gastropods are aggregated on the carrion.

where: L_∞ = asymptotic maximum length

K= growth rate

t_0= the nominal age at which length is equal to zero

C = the relative amplitude of the seasonal oscillations in growth rate

t_S = phase of seasonal oscillations

The parameter C can take values from 0 to 1 and it is correlated with the difference between mean monthly summer and winter air or water temperature (Pauly *et al.*, 1992). When there is no difference between summer and winter seawater temperature, there is no seasonal variation in growth rate (C=0), and the above equation becomes the simpler non-seasonal version of the von Bertalanffy equation. The higher the value of C, the more pronounced is the seasonal variation. When summer and winter temperatures fluctuate by more than 10°C, then C is equal to unity and growth actually ceases for a period during the year. The parameter t_S can take values from –0.5 to 0.5 and it corresponds to the start of the convex segment of sinusoidal oscillation. The time of the year when growth rate is the slowest is equal to $t_S + ½$ and it is more often known as the winter point (WP).

In situations where shell growth stops for a period during the year (C=1) the Pauly model (Pauly *et al.*, 1992) was used to describe the growth of the population, according to the formula: $L(t) = L_\infty(1-\exp(-q))$

where: $$q = K(t'-t_0)+\frac{K}{Q}[\sin Q(t'-t_S)]-\frac{K}{Q}[\sin Q(t_0-t_S)]$$

$$Q=\frac{2\pi}{1-NGT}$$

$t' = t_0$ for all $t \leq t_0$, and $t' = t_0$ + total growth time between t_0 and t, for $t \geq t_0$

The parameter NGT (0≤NGT<1) measures the length of time when no growth occurs. The no growth period commenses at time t_S + ½ - ½ NGT and finishes at time t_S + ½ + ½ NGT each year.

The length-frequency distributions obtained during the monthly sampling of *Nassarius reticulatus* from the intertidal lagoon and subtidal beach populations were analysed using two different approaches.

1) A Modal Progression Analysis (MPA) was followed where growth was inferred from the apparent shift of mean sizes in a time series of length-frequency data (FISAT II, version 1.2.0, FAO-ICLARM). The MPA involves three stages: 1) decomposition of length-frequency distributions into their components in order to identify the mean shell length of each cohort based on Bhattacharya's (1967) method, 2) identification and linking of the means perceived to belong to the same cohorts and 3) use of the growth increments that result from the mean linking in order to estimate the von Bertalanffy growth parameters based on Appeldoorn's (1987) method.

Following Appeldoorn's method, FISAT II estimates the growth parameters (L_∞, K, C and t_S) by minimising the following function using the Marquardt's algorithm for a non-linear fit: $SSE = \sum \left[L_{i+\Delta t} - \left(L_\infty - \left(L_\infty - L_t\right)\exp\left(-\left(K\Delta_t - S_t + S_{t+\Delta t}\right)\right)\right)\right]^2$

where: $S_t = \left(\frac{CK}{2\pi}\right)\sin\left(2\pi\left(t - t_S\right)\right)$

$$S_{t+\Delta t} = \left(\frac{CK}{2\pi}\right)\sin\left(2\pi\left(\left(t + \Delta t\right) - t_S\right)\right)$$

2) The LFDA analysis is a computerised method that generates a growth curve from the "best fit" to the length-frequency data and calculates the von Bertalanffy growth parameters using the ELEFAN method (LFDA, version 5.0, MRAG Ltd). A wide range of L_∞ and K values was found initially and was gradually reduced in order to maximise the goodness of fit of the curves to the data (Kirkwood *et al.*, 2001). After establishing the L_∞ and K values with the maximum goodness of fit, a range of C and t_S values was also included in the model. Again the range of C and t_S values was gradually reduced until the maximum goodness of fit was obtained. Finally all the parameters of the von Bertalanffy growth curve with the best fit on the original length-frequency data were identified for the intertidal lagoon and the subtidal beach populations.

The estimation of the total mortality rate (Z) of *Nassarius reticulatus* from the intertidal lagoon and from the subtidal beach was based on the Beverton & Holt (1956) model

(FISAT II, version 1.2.0 FAO-ICLARM) and was calculated according to the following formula:

$$Z = \frac{K(L_\infty - L_{mean})}{L_\infty - L'}$$

where L_∞ and K are the growth parameters from the von Bertalanffy growth curve, L' is the cut-off length (the smallest length) and L_{mean} is the mean length. The percentage of % survival rate per annum was calculated as $e^{-Z} \cdot 100$.

3.2.3 Growth studies under laboratory conditions

3.2.3.1 Comparison of growth rates between different size classes, different seasons, different sexes and different origins

A group of 77 *Nassarius reticulatus* (8.9-28.9 mm) collected from the intertidal lagoon in May 2002 and a group of 100 gastropods (7.6-28.6 mm) collected from the subtidal beach in June 2002, were transferred to the laboratory. They were divided into groups of 5-6 animals according to their size-class and placed in small plastic tanks (L:17 x H:10 x D:11cm) containing a 3 cm layer of shell-sand sediment. The tanks were provided with running seawater at ambient temperature. Weekly half of a freshly opened mussel was provided to the whelks in each small tank. In order to monitor the growth of each individual, each whelk was marked with a small numbered tag fixed to its shell with superglue. The shell length of each *N. reticulatus* was measured to the nearest 0.1 mm with vernier callipers at the start of the experiment and then re-measured once every month until December 2002. The seawater temperature in one of the small tanks was continuously monitored with a Tinytalk II temperature logger. At the end of the experiment the shells of the whelks were cracked open, and the sex of each individual identified after dissection (for details of sex identification methods see chapter 7).

The growth rate ($R\%$) of individuals of different size classes was estimated during different seasons for females and males originating from the intertidal lagoon or from the subtidal beach using the equation: $R\% = \frac{\ln L_1 - \ln L_0}{t} \cdot 100$, where $R\%$ is the relative daily growth rate, L_1 is the shell length (mm) after a time period t (days) and L_0 is the initial shell length (mm).

3.2.3.2 Effect of limited food supply on growth of *Nassarius reticulatus* held under laboratory conditions

A second laboratory growth experiment was conducted in order to study the effects of starvation on shell growth of *Nassarius reticulatus*. One hundred and twelve whelks were collected from the intertidal lagoon in March 2004 and maintained in the laboratory under similar conditions to those described above. During the experiment the whelks were not individually tagged. One group (56) (9.7-28.1 mm) of the experimental animals were fed as usual with half of a freshly opened mussel once a week ("feeding" treatment), whilst the other group (56) (10.1-27.5 mm) were fed only once a month ("starvation" treatment). Shell length and shell lip thickness were measured with vernier callipers to the nearest 0.1 mm monthly from March until August 2004. The growth patterns of the regularly weekly fed whelks and those fed less often were compared.

3.2.4 Release and recapture of marked animals into the intertidal lagoon

Five hundred and sixty six *Nassarius reticulatus* of a range of lengths (7.9-31.4 mm) were collected from the intertidal lagoon. Each whelk was labelled individually with a small numbered tag fixed to its shell with superglue and their initial shell length measured. The whelks were released back into the lagoon during three different releases: 165 were released in June 2002, 101 in September 2002 and 300 in May 2003 (see figure 3.3). The animals were recaptured opportunistically during the routine monthly sampling, their shell size was re-measured and they were once again returned into the intertidal lagoon.

"Jackson's Positive Method" (Jackson, 1939) was used to estimate the size of the intertidal lagoon population from the marked and recaptured whelks as described by Begon (1979). This method can be applied in cases when a single marking occasion and several recaptures take place and allows an estimation of the population size (N_0) at the first sampling date and also an estimation of the birth rate (b). The birth rate is also called gain rate because it actually represents all the new additions in the population without distinguishing between birth and immigration. "Jackson's Positive Method" allows a degree of loss (as death and emigration) within the population at a variable rate, and assumes that gain is constant.

Figure 3.3 *Nassarius reticulatus* marked individually with numbered tags and released in the intertidal lagoon during May 2003.

On the start date (i=0) a number of r_0 gastropods are collected, marked and released. During the following dates of recapture (i =1,2,3,4...) a number of n_i gastropods are collected from which m_i are marked. The proportion of marked animals (q_i) at date i is given by the equation $q_i = \frac{m_i}{n_i}$. The proportion of marked animals q_i is reduced during the subsequent recaptures because the number of marked animals remains the same, while the number of unmarked animals increases due to the appearances of new recruits into the population. The size of the population N_0 at the date of first release (i=0) can be estimated from the formula $N_0 = \frac{r_0}{q_0}$. The proportion of marked animals in the population q_0 at the starting date is calculated from the formula $\ln q_0 = \overline{\ln q} - \ln(1-b)\bar{i}$, where b is the birth rate, $\overline{\ln q}$ is the average value for all the $\ln q_i$ and $\bar{i}$ is the average value for all the dates i. The birth or gain rate b can be calculated from the equation:

$$\ln(1-b) = \frac{\sum m_i(\ln q_i - \overline{\ln q})(i-\bar{i})}{\sum m_i(i-\bar{i})^2}$$

The standard errors of $\ln(1-b)$ and $\ln q_0$ are given by the following equations, where n is the number of recaptures.

$$SE_{\ln(1-b)} = \sqrt{\frac{\sum m_i \left[\ln q_i - \overline{\ln q} - \ln(1-b)(i-\bar{i})^2\right]}{(n-2)\sum m_i (i-\bar{i})^2}}$$

$$SE_{\ln q_0} = \sqrt{\frac{\sum m_i \left[\ln q_i - \overline{\ln q} + \bar{i} \cdot \ln(1-b)\right]^2}{(n-1)\sum m_i}}$$

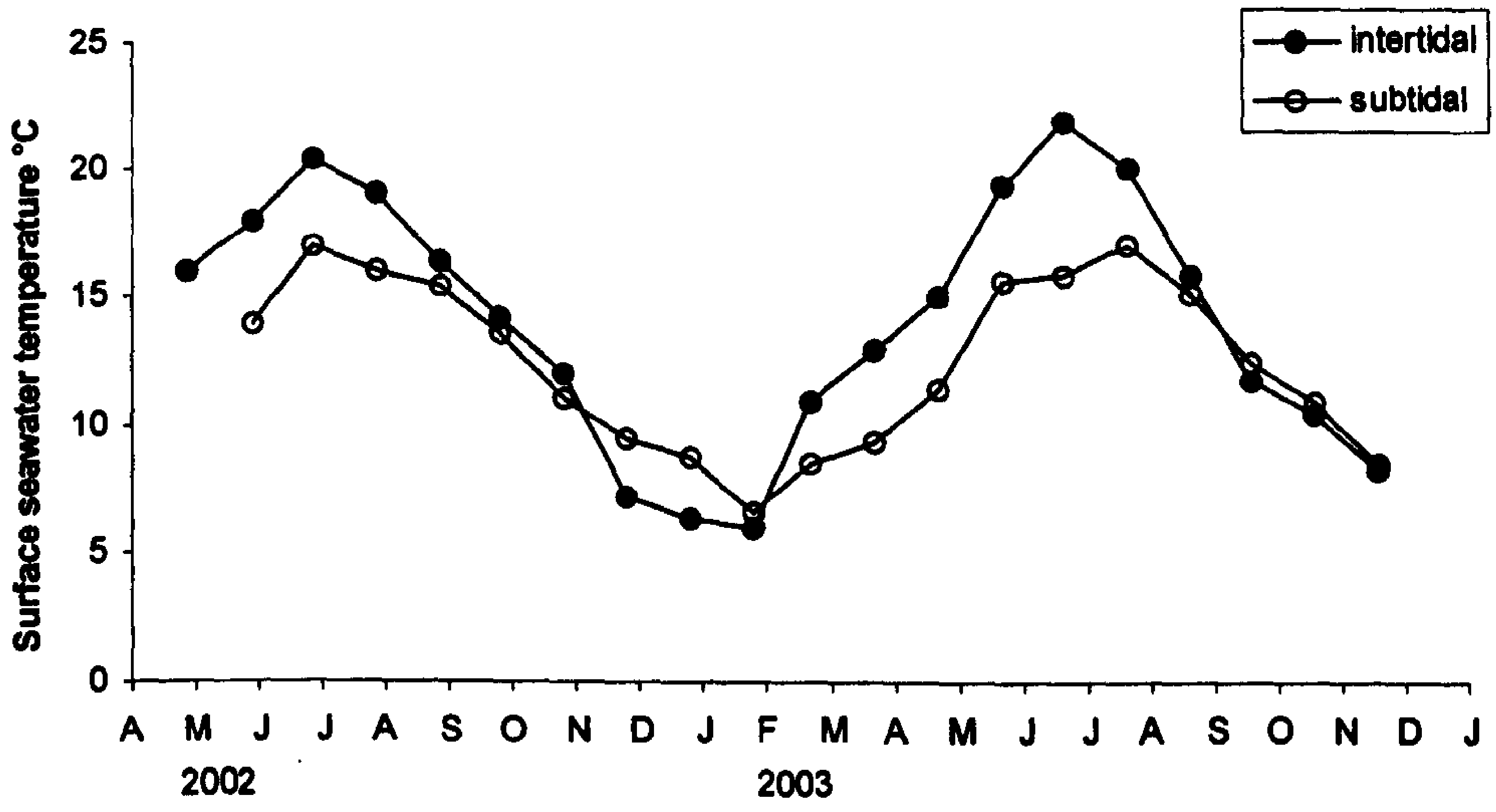
The growth of the marked and recaptured *Nassarius reticulatus* in the intertidal lagoon was compared with the growth of the laboratory maintained animals that were regularly fed (see 3.2.3.1). In addition, the mark-recapture growth data were used to evaluate the validity of the growth patterns described using the MPA and LFDA methods (see 3.2.2). Measurements of the growth increments from the mark-recapture whelks were analysed according to Munro's method (Munro, 1982) for estimating the growth parameters L_∞ and K in the von Bertalanffy equation. The parameters were calculated from the FISAT software by minimising the coefficient of variation of the ratio $\frac{\left[\ln(L_\infty - L_m) - \ln(L_\infty - L_r)\right]}{(t_r - t_m)}$ where L_m is the length at marking, L_r is the length at recapture and t_m and t_r are the corresponding dates. Munro's method does not account for seasonal growth oscillations.

3.3 RESULTS

3.3.1 Surface seawater temperature and sediment type

The seasonal variation in surface seawater temperature (SST) from May 2002 to December 2003 for both the Rhosneigr intertidal lagoon and beach is shown in figure 3.4. Similar temperature fluctuations were observed at both sites, however, in the intertidal lagoon the temperature was about 3 to 6°C higher during the spring and summer months (March-August), and 2.4 °C lower during winter (December-January). The sediment on the Rhosneigr beach was fine sand (mean=2.58 Ø) moderately sorted (St. Dev.=0.95 Ø), whereas in the intertidal lagoon it was coarse sand (mean=0.29 Ø) and poorly sorted (St.Dev.=1.65 Ø). The percentage of silt content was 1.132% for the beach and 0.623% for the lagoon sediment. The percentage of organic content was 0.363% and 0.654% for the beach and lagoon sites respectively.

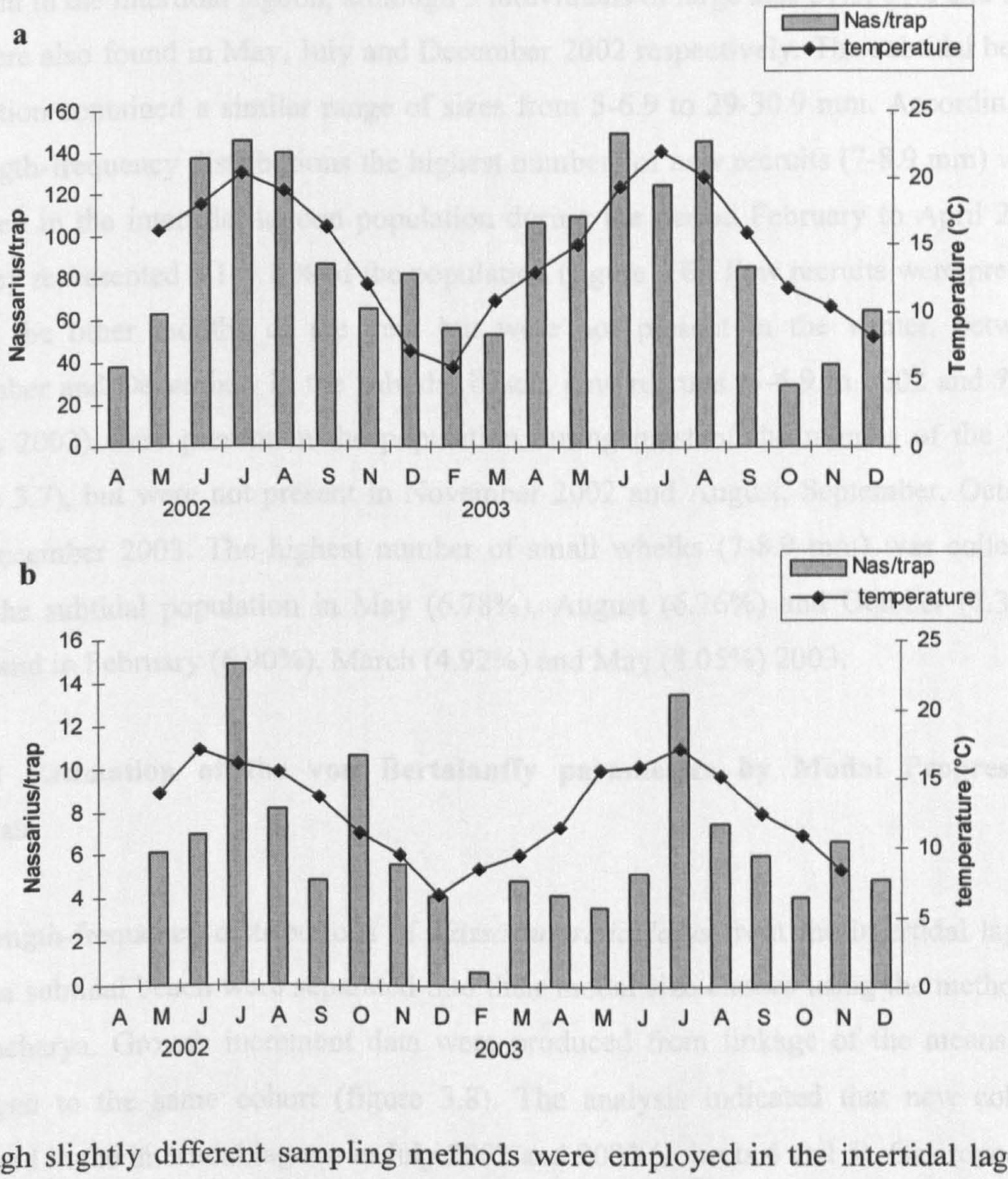
Figure 3.4 Seasonal variation in surface seawater temperature (°C) in the Rhosneigr intertidal lagoon and subtidal beach sites.



3.3.2 Seasonal variation in abundance of *Nassarius reticulatus*

The abundance of *Nassarius reticulatus* was estimated as catchability per unit effort, i.e. the number of whelks caught per trap during a 30 minute period. The seasonal variation in catchability of *N. reticulatus* in the Rhosneigr intertidal lagoon and the beach are shown in figure 3.5. A significant correlation was found between the catchability of *N. reticulatus* and SST for both the intertidal lagoon (Pearson =0.787, p<0.001) and the subtidal beach (Pearson =0.675, p=0.002). In 2002 the catchability of *N. reticulatus* in the intertidal lagoon was significantly higher during the summer months June, July and August (137, 145.3 and 140 *Nas*/trap respectively) when the SST was at its warmest (18, 20.4 and 19°C respectively). Similarly in 2003 the highest catchability in the intertidal lagoon was observed during June, July and August (150, 124.8 and 145.5 *Nas*/trap respectively) when again the SST was warmest (19.4, 22 and 20.1°C respectively). Lower numbers of *N. reticulatus* were collected per trap in the intertidal lagoon during the colder months February to March in 2003 (56.5 *Nas*/trap at 5.97°C and 53.6 *Nas*/trap at 10.9°C respectively) and October to December in 2003 (29.7 *Nas*/trap at 11.8°C, 39 *Nas*/trap at 10.4°C and 64.8 *Nas*/trap at 8.2°C respectively). The highest number of *N. reticulatus* on the subtidal beach were observed during July 2002 and 2003 (14.9 and 13.6 *Nas*/trap respectively) when the SST was 17°C and 15.9°C respectively. Fewer *Nassarius* were collected per trap (≤5 *Nas*/trap) on the subtidal beach during the winter and early spring months (December to April) when the SST fell below 9.5°C. The lowest number of *Nassarius* per trap on the subtidal beach was collected in February 2003 (0.6 *Nas*/trap) when the SST was just 6.6°C.

Figure 3.5 Seasonal variation in the catchability of *Nassarius reticulatus* (*Nassarius* collected per trap in a period of 30 minutes, Nas/trap) and seawater temperature (°C) (a) in the intertidal lagoon and (b) in the subtidal beach at Rhosneigr. Note the different scales on the Y axis (*Nassarius*/trap) in (a) and (b).



Although slightly different sampling methods were employed in the intertidal lagoon and the subtidal beach, the traps were deployed for the same time (30 minutes), thus comparison of catchability per unit of effort time is a valid way for evaluating differences in the abundance of gastropods between the two habitats. A large difference in the number of *Nassarius* collected per trap was observed between the intertidal lagoon and the subtidal beach. Even the highest catchability of *Nassarius* from the beach (July 2002 and 2003, 14.9 and 13.6 *Nas*/trap respectively), was lower than the lowest catchability of whelks from the intertidal lagoon (October 2003, 29.7 *Nas*/trap).

3.3.3 Population structure of *Nassarius reticulatus*

The shell length-frequency histograms are presented in figure 3.6 for the intertidal lagoon and in figure 3.7 for the subtidal beach. Whelks ranged in size from 7-8.9 to 29-30.9 mm in the intertidal lagoon, although 3 individuals of large size 31.5, 31.8 and 33.2 mm were also found in May, July and December 2002 respectively. The subtidal beach population contained a similar range of sizes from 5-6.9 to 29-30.9 mm. According to the length-frequency distributions the highest numbers of new recruits (7-8.9 mm) were observed in the intertidal lagoon population during the period February to April 2003 and they represented 2.1-5.1 % of the population (figure 3.6). Few recruits were present during the other months of the year but were not present in the winter, between November and December. In the subtidal beach, new recruits (5-6.9 in 2002 and 7-8.9 mm in 2003) were present in the population during most of the months of the year (figure 3.7), but were not present in November 2002 and August, September, October and December 2003. The highest number of small whelks (7-8.9 mm) was collected from the subtidal population in May (6.78%), August (6.26%) and October (7.38%) 2002, and in February (6.90%), March (4.92%) and May (8.05%) 2003.

3.3.3.1 Estimation of the von Bertalanffy parameters by Modal Progression Analysis

The length-frequency distributions of *Nassarius reticulatus* from the intertidal lagoon and the subtidal beach were separated into their modal size classes using the method of Bhattacharya. Growth increment data were produced from linkage of the means that belonged to the same cohort (figure 3.8). The analysis indicated that new cohorts appeared in the intertidal lagoon in July 2002 and 2003 (cohorts 4 and 5). Gastropods of about 6 mm size in July 2002 reached a size of 16.6 mm by July 2003 (cohort 4). Gastropods of 14.9 mm shell length in September 2002 had grown to a size of 21 mm one year later (cohort 3), and gastropods of 20 mm in August 2002 (cohort 2) were part of the higher size class (cohort 1) a year later with a size of 25.4 mm.

Figure 3.6 Seasonal variation in the shell length-frequency distribution of *Nassarius reticulatus* from the intertidal lagoon in Rhosneigr between April 2002 and December 2003. The total number of whelks collected (N) is also indicated. No attempt has been made in this figure to identify visually the cohorts (age classes) within the population, but instead the Bhattacharya's method was used in the MPA analysis (see figure 3.8a).

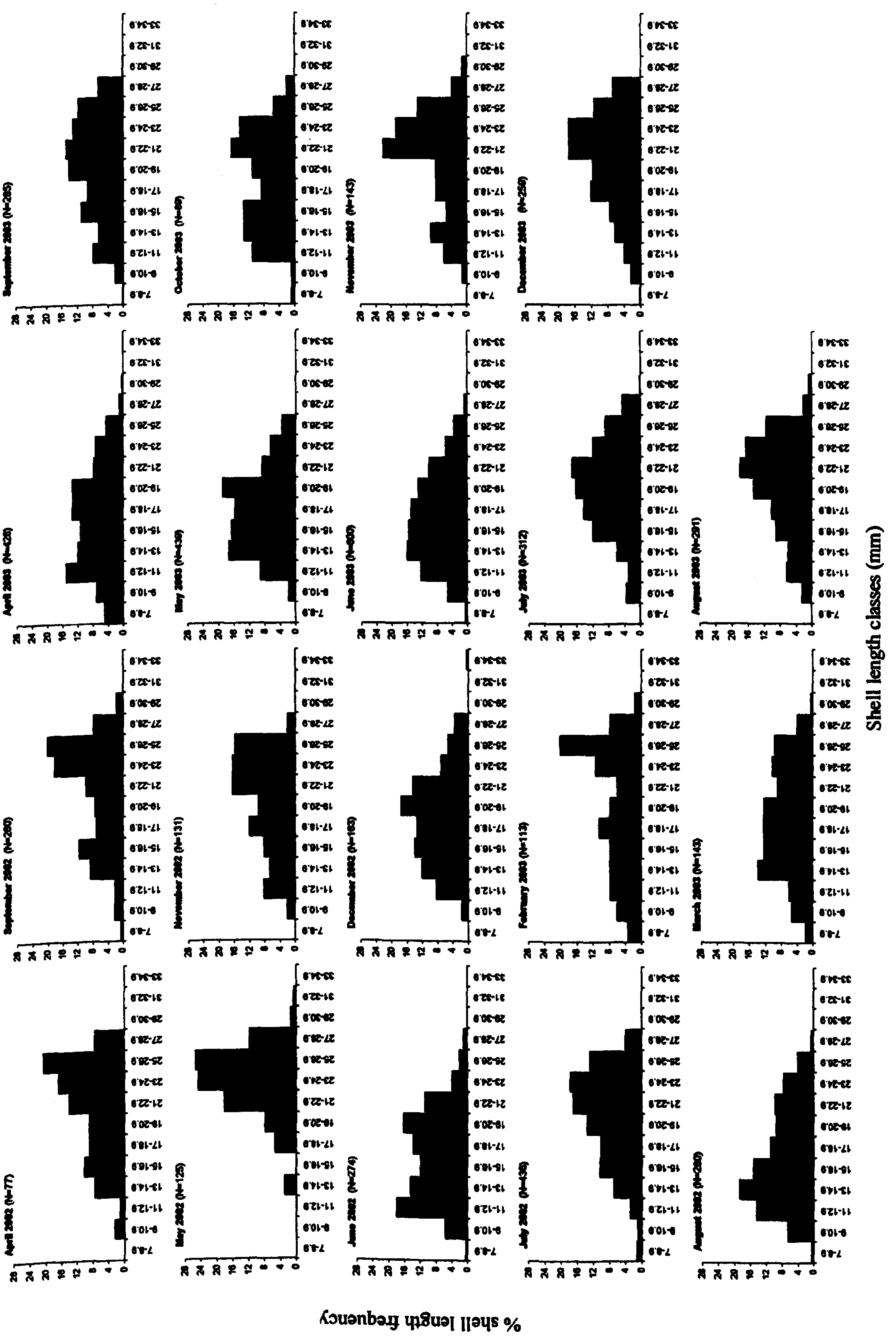
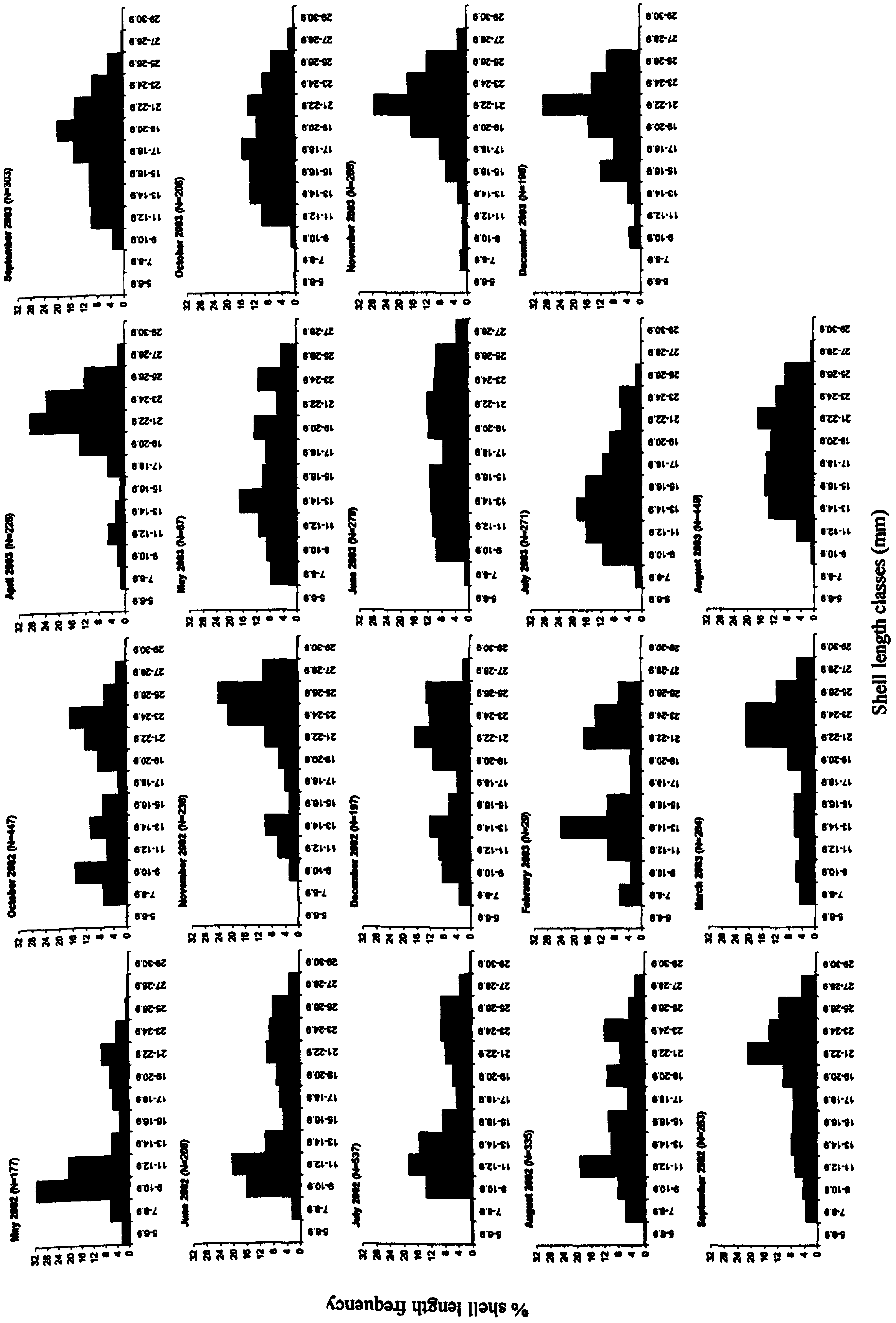
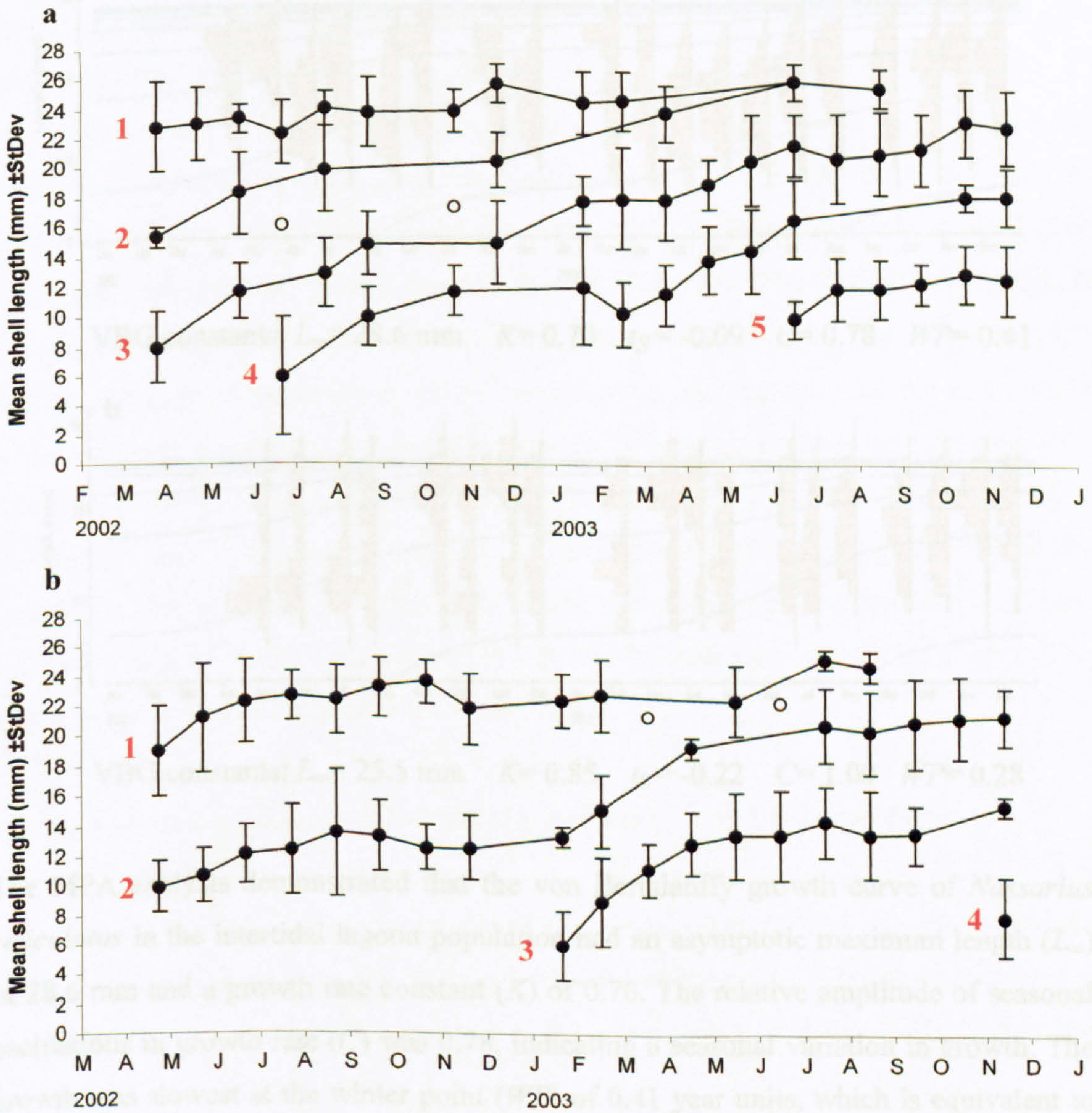

April 2002 (N=77)
May 2002 (N=125)
June 2002 (N=274)
July 2002 (N=438)
August 2002 (N=260)
September 2002 (N=260)
November 2002 (N=131)
December 2002 (N=163)
February 2003 (N=113)
March 2003 (N=143)
April 2003 (N=426)
May 2003 (N=439)
June 2003 (N=600)
July 2003 (N=312)
August 2003 (N=291)
September 2003 (N=285)
October 2003 (N=89)
November 2003 (N=143)
December 2003 (N=259)
0
4
8
12
16
20
24
28
7-8.9
9-10.9
11-12.9
13-14.9
15-16.9
17-18.9
19-20.9
21-22.9
23-24.9
25-26.9
27-28.9
29-30.9
31-32.9
33-34.9
% shell length frequency
Shell length classes (mm)

Figure 3.7 Seasonal variation in the shell length-frequency distribution of *Nassarius reticulatus* from the subtidal beach in Rhosneigr between May 2002 and December 2003. The total number of whelks collected (N) is also indicated. No attempt has been made in this figure to identify visually the cohorts (age classes) within the population, but instead the Bhattacharya's method was used in the MPA analysis (see figure 3.8b).


May 2002 (N=177)
June 2002 (N=208)
July 2002 (N=537)
August 2002 (N=335)
September 2002 (N=263)
October 2002 (N=447)
November 2002 (N=238)
December 2002 (N=197)
February 2003 (N=29)
March 2003 (N=294)
April 2003 (N=226)
May 2003 (N=87)
June 2003 (N=278)
July 2003 (N=271)
August 2003 (N=448)
September 2003 (N=303)
October 2003 (N=205)
November 2003 (N=286)
December 2003 (N=196)
0
4
8
12
16
20
24
28
32
5-6.9
7-8.9
9-10.9
11-12.9
13-14.9
15-16.9
17-18.9
19-20.9
21-22.9
23-24.9
25-26.9
27-28.9
29-30.9
% shell length frequency
Shell length classes (mm)

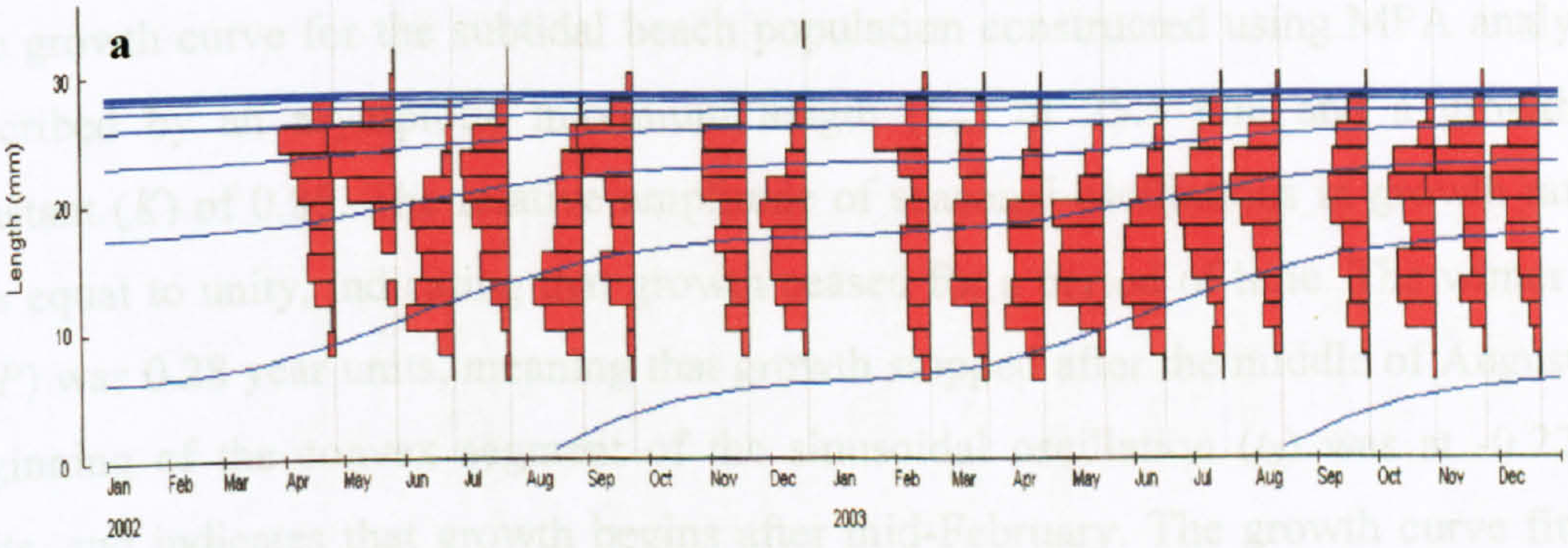
New cohorts (6 mm) appeared in the subtidal beach population during February 2003 and reached a size of 13.5 by the end of the growing season in October 2003 (cohort 3). Gastropods of cohort 2 were 10 mm in May 2002, 13.3 mm size in February 2003, and had a mean shell length of 20.9 mm by October 2003. Growth of the older cohort 1 was slower with 19.0 mm whelks in May 2002 reaching a size of 22.4 mm in February 2003 and a size of 24.8 mm by September 2003.

Figure 3.8 Identification of mean shell length (±StDev) of all cohorts in each monthly sample and construction of growth curves by linking the means that belong to the same cohort from (a) the intertidal lagoon and (b) the subtidal beach. The open circles are means that were not used for the construction of growth curves because they fell between cohorts 2 and 3 in (a) and cohorts 1 and 2 in (b).

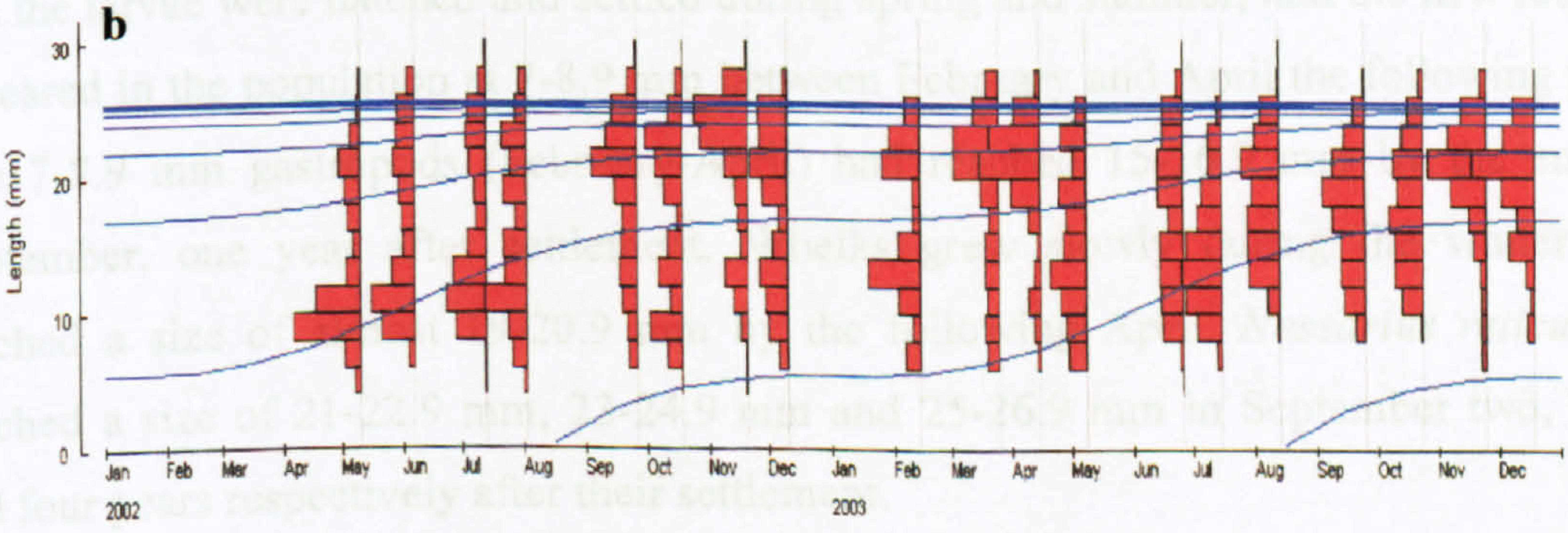


The parameters of the von Bertalanffy growth curves were estimated from the growth increment data of the MPA analysis based on Appeldoorn's method using the FISAT software (figure 3.9). The parameters t_S and *WP* are "year units" and the beginning of a "year" is equivalent to the spawning period. The spawning period of *Nassarius reticulatus* begins in May and eggs are usually laid at Rhosneigr and in the laboratory following this month (see chapter 5).

Figure 3.9 Von Bertalanffy seasonal growth curves fitted to the length-frequency data of the *Nassarius reticulatus* from (a) the intertidal lagoon and (b) the subtidal beach populations. Curves are constructed from the growth parameters determined after using the MPA analysis.



VBG constants: L_∞= 28.6 mm K= 0.70 t_S= -0.09 C= 0.78 WP= 0.41



VBG constants: L_∞= 25.5 mm K= 0.85 t_S= -0.22 C= 1.00 WP= 0.28

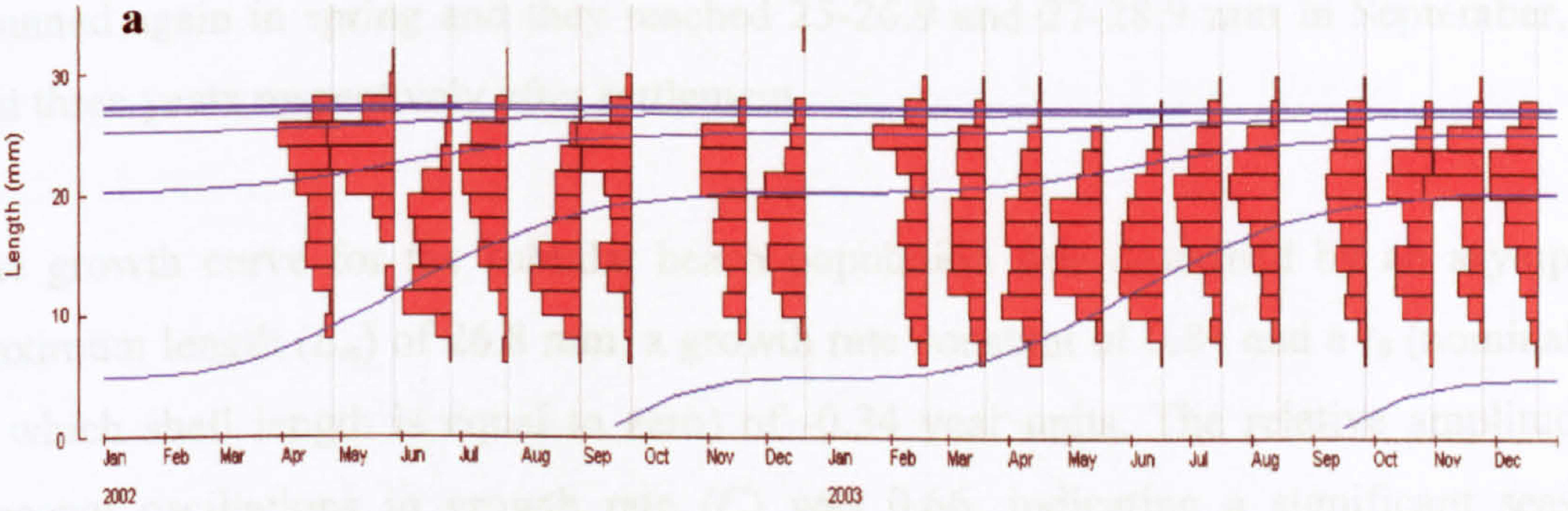
The MPA analysis demonstrated that the von Bertalanffy growth curve of *Nassarius reticulatus* in the intertidal lagoon population had an asymptotic maximum length (L_∞) of 28.6 mm and a growth rate constant (K) of 0.70. The relative amplitude of seasonal oscillations in growth rate (C) was 0.78, indicating a seasonal variation in growth. The growth was slowest at the winter point (*WP*) of 0.41 year units, which is equivalent to the end of September. The beginning of the convex segment of the sinusoidal oscillation (t_S) was at -0.09 year units, indicating that shell growth rates begin to increase at the beginning of April. The growth curve constructed using MPA analysis and fitted to the length-frequency data for *N. reticulatus* from the intertidal population indicated that the larvae were hatched and settled during spring and summer, and the new recruits appeared as part of the population at 7-8.9 mm between February and April the following year. The 7-8.9 mm *N. reticulatus* (February-April) had reached a size of 15-16.9 mm by the end of September one year after settlement. Growth did not cease completely during winter and the whelks continued to grow slowly up to 19-20.9 mm the following April. *N. reticulatus* reached a size of 23-24.9 mm, 25-26.9 mm and 27-28.9 mm in September, two, three and four years respectively after their settlement.

The growth curve for the subtidal beach population constructed using MPA analysis is described by an asymptotic maximum length (L_∞) of 25.5 mm and a growth rate constant (K) of 0.85. The relative amplitude of seasonal oscillations in growth rate (C) was equal to unity, indicating that growth ceased for a period of time. The winter point (WP) was 0.28 year units, meaning that growth stopped after the middle of August. The beginning of the convex segment of the sinusoidal oscillation (t_S) was at -0.22 year units, and indicates that growth begins after mid-February. The growth curve fitted to the length-frequency data after the MPA analysis for the subtidal population suggests that the larvae were hatched and settled during spring and summer, and the new recruits appeared in the population at 7-8.9 mm between February and April the following year. The 7-8.9 mm gastropods (February-April) had reached 15-16.9 mm by the end of September, one year after settlement. Whelks grew slowly during the winter and reached a size of almost 19-20.9 mm by the following April. *Nassarius reticulatus* reached a size of 21-22.9 mm, 23-24.9 mm and 25-26.9 mm in September two, three and four years respectively after their settlement.

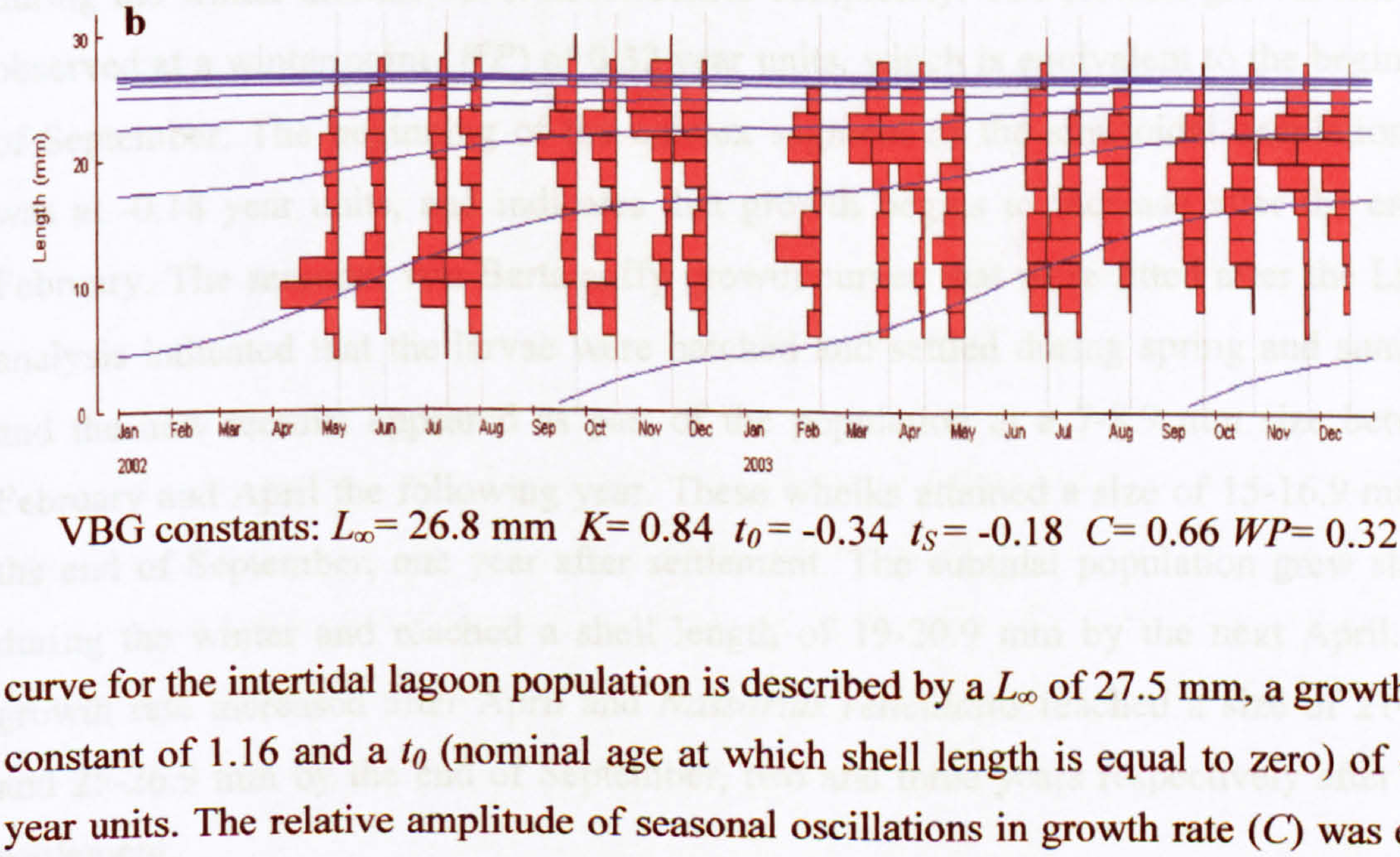
3.3.3.2 Estimation of the von Bertalanffy parameters by LFDA analysis

The von Bertalanffy curves and growth constants determined for the *Nassarius reticulatus* population from the intertidal lagoon and the subtidal beach were generated from the computerised LFDA method and are presented in figure 3.10. The growth

Figure 3.10 Von Bertalanffy seasonal growth curves fitted to the length-frequency data obtained from (a) the intertidal *Nassarius reticulatus* lagoon population and (b) the subtidal beach population. Curves are constructed from the growth parameters determined after using the LFDA analysis.



VBG constants: L_∞= 27.5 mm K= 1.16 t_0= -0.87 t_S= 0.08 C= 1.00 WP= 0.58



VBG constants: L_∞= 26.8 mm K= 0.84 t_0= -0.34 t_S= -0.18 C= 0.66 WP= 0.32

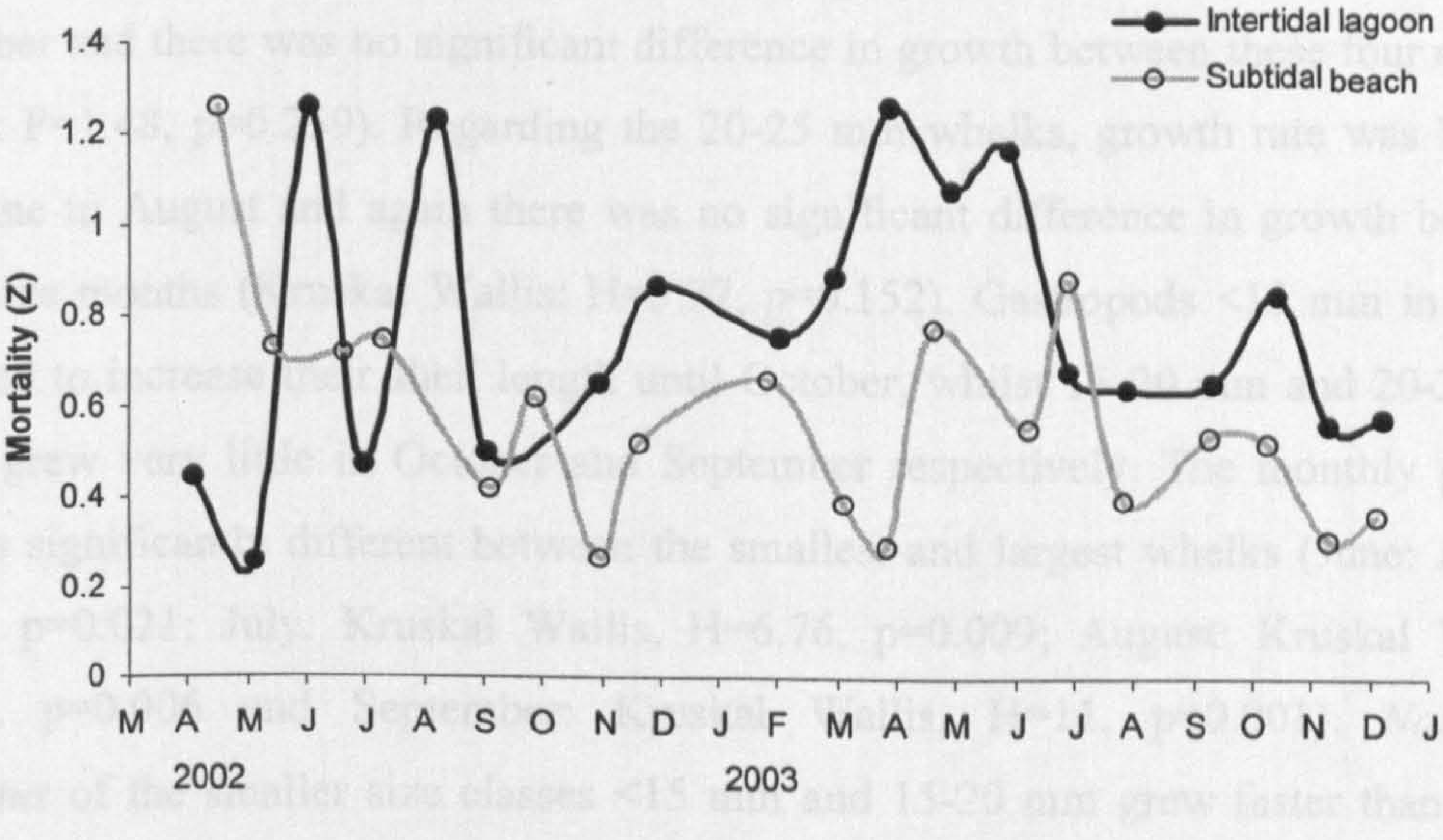
curve for the intertidal lagoon population is described by a L_∞ of 27.5 mm, a growth rate constant of 1.16 and a t_0 (nominal age at which shell length is equal to zero) of -0.87 year units. The relative amplitude of seasonal oscillations in growth rate (C) was equal to unity, indicating a very strong seasonal variation in growth. The growth rate of *Nassarius* in the intertidal lagoon is slower during the winter months and ceases completely at a winter point (WP) of 0.58 year units, which is equivalent to the end of November. The "no growth period" (NGT) actually commences at 0.51 and finishes at 0.66 year units, signifying that growth of whelks in the lagoon ceased between the beginning of November and the middle of December. The beginning of the convex segment of the sinusoidal oscillation (t_S) was at 0.08 year units, which is equivalent to the end of May, and indicates the period of the year when growth starts to increase. The von Bertalanffy growth curves that were fitted after the LFDA analysis suggest that the larvae hatched and settled during spring and summer, and the new recruits appeared as part of the population at 7-8.9 mm between February and April the following year. The 7-8.9 mm gastropods (February-April) reached 19-20.9 mm by the end of September, one year after settlement. Individuals grew very little during winter because of the zero growth period, and they attained a size of 21-22.9 mm by the following April. Growth resumed again in spring and they reached 25-26.9 and 27-28.9 mm in September, two and three years respectively after settlement.

The growth curve for the subtidal beach population was described by an asymptotic maximum length (L_∞) of 26.8 mm, a growth rate constant of 0.84 and a t_0 (nominal age at which shell length is equal to zero) of -0.34 year units. The relative amplitude of seasonal oscillations in growth rate (C) was 0.66, indicating a significant seasonal variation in growth. The growth rate of *Nassarius* from the subtidal beach slows down during the winter months but it never ceases completely. The slowest growth rate was observed at a winter point (WP) of 0.32 year units, which is equivalent to the beginning of September. The beginning of the convex segment of the sinusoidal oscillation (t_S) was at -0.18 year units, and indicates that growth begins to increase after the end of February. The seasonal von Bertalanffy growth curves that were fitted after the LFDA analysis indicated that the larvae were hatched and settled during spring and summer, and the new recruits appeared as part of the population at a 7-8.9 mm size between February and April the following year. These whelks attained a size of 15-16.9 mm by the end of September, one year after settlement. The subtidal population grew slowly during the winter and reached a shell length of 19-20.9 mm by the next April. The growth rate increased after April and *Nassarius reticulatus* reached a size of 21-22.9 and 25-26.9 mm by the end of September, two and three years respectively after their settlement.

3.3.3.3 Mortality of *Nassarius reticulatus*

Estimates of the monthly total mortality rate of *Nassarius reticulatus* from the lagoon and the subtidal beach were calculated using the Beverton & Holt (1956) method (figure 3.11). The total mortality rate (Z) represents the number of individuals that die during a season (month, year) in relation to the initial size of the population. The annual rate of total mortality of the *N. reticulatus* was 0.778 for the intertidal lagoon and 0.577 for the subtidal beach. Since there is no fishing mortality of *N. reticulatus* in Rhosneigr, the total mortality is equal to the natural mortality of the population. The percentage survival rate per annum was 45.9% for the lagoon population and 56.2% for the subtidal beach population.

Figure 3.11 Seasonal variation in total mortality rate of the *Nassarius reticulatus* populations from the intertidal lagoon (closed circles) and the subtidal beach (open circles) in Rhosneigr.



A similar seasonal pattern of mortality was observed between the intertidal lagoon and the subtidal beach habitats, although mortality was higher in the intertidal lagoon than in the subtidal beach. Mortality was greatest during the late spring and early summer and lower during the autumn and winter, as is particularly clear for the intertidal lagoon population.

3.3.4 Growth under laboratory conditions

3.3.4.1 Comparison of growth rates of different size classes during different seasons

Nassarius reticulatus from the intertidal lagoon and the subtidal beach were maintained in the laboratory between June-December 2002 and July-December 2002 respectively.

In order to eliminate any possible effect of sex or origin on growth, the monthly growth rate (R%) of individuals was calculated separately for female and male whelks, originating from the lagoon or the subtidal beach (table 3.1). Whelks of same sex and origin were grouped into 4 size classes <15mm, 15-20mm, 20-25mm and >25mm.

Females originating from the lagoon increased in shell length between June and September when seawater temperatures were higher (14.4-17°C), while growth ceased during the winter months between October and December (seawater temperatures 7.8-13.1°C). Growth of the <15 mm and 15-20 mm gastropods was highest from June to September and there was no significant difference in growth between these four months (Anova: F=1.48, p=0.259). Regarding the 20-25 mm whelks, growth rate was highest from June to August and again there was no significant difference in growth between these three months (Kruskal Wallis: H=3.77, p=0.152). Gastropods <15 mm in length continued to increase their shell length until October, whilst 15-20 mm and 20-25 mm whelks grew very little in October and September respectively. The monthly growth rate was significantly different between the smallest and largest whelks (June: Anova, F=6.51, p=0.021; July: Kruskal Wallis, H=6.76, p=0.009; August: Kruskal Wallis, H=7.65, p=0.006 and September: Kruskal Wallis, H=11, p=0.001). *Nassarius reticulatus* of the smaller size classes <15 mm and 15-20 mm grew faster than larger size classes (20-25 mm). Individuals >25 mm did not increase their shell length at any time.

Males originating from the lagoon increased in shell length during the warmer months from June to September, while growth ceased during the winter months from October to December. In particular, growth rate was higher from June to September for whelks <15 mm and there was no significant difference in the growth rate between these 4 months (Anova: F=0.67, p=0.588). For 15-20 mm whelks growth rate was higher between June and August and there was no significant difference in the growth rate between these three months (Kruskal Wallis: H=1.18, p=0.554). Growth of 20-25 mm whelks was higher in June and July and there was no significant difference between the two months (Kruskal Wallis: H=0.05, p=0.824). Animals <15 mm continued to increase their shell length until October, while those of 15-20 mm and 20-25 mm had a low growth rate in September and August respectively. *Nassarius reticulatus* >25 mm did not increase their shell length at any time. The monthly growth rates were significantly different

Table 3.1 Mean monthly growth rate (mm·d^{-1}) (R%) (±SE) of different size classes between June and December 2002 for female and male *Nassarius reticulatus* collected from the intertidal lagoon and the subtidal beach in Rhosneigr and maintained under laboratory conditions. The size of the sample (N) is also indicated. n.a.: non-applicable, number of samples=1, n.g.: no growth

	Size class	N	June	July	August	September	October	November	December
Intertidal lagoon	<15 mm	1	0.47 (n.a.)	0.47 (n.a.)	0.73 (n.a.)	0.70 (n.a.)	0.23 (n.a.)	n.g.	n.g.
Females	15-20 mm	5	0.34 (±0.07)	0.36 (±0.04)	0.38 (±0.07)	0.22 (±0.05)	0.04 (±0.02)	n.g.	n.g.
	20-25 mm	13	0.16 (±0.04)	0.16 (±0.04)	0.09 (±0.03)	0.02 (±0.02)	0.01 (±0.01)	n.g.	n.g.
	>25 mm	14	0.01 (±0.01)	n.g.	n.g.	n.g.	n.g.	n.g.	n.g.
Intertidal lagoon	<15 mm	4	0.35 (±0.03)	0.40 (±0.04)	0.40 (±0.04)	0.35 (±0.03)	0.13 (±0.05)	n.g.	n.g.
Males	15-20 mm	6	0.32 (±0.05)	0.38 (±0.03)	0.32 (±0.05)	0.08 (±0.04)	n.g.	n.g.	n.g.
	20-25 mm	11	0.10 (±0.03)	0.09 (±0.03)	0.03 (±0.02)	n.g.	n.g.	n.g.	n.g.
	>25 mm	2	n.g.	n.g.	n.g.	n.g.	n.g.	n.g.	n.g.
Subtidal beach	<15 mm	21		0.86 (±0.05)	0.59 (±0.03)	0.26 (±0.03)	0.06 (±0.02)	0.01 (±0.01)	n.g.
Females	15-20 mm	6		0.45 (±0.09)	0.38 (±0.03)	0.05 (±0.03)	n.g.	n.g.	n.g.
	20-25 mm	17		0.12 (±0.04)	0.08 (±0.03)	n.g.	n.g.	n.g.	n.g.
	>25 mm	4		n.g.	n.g.	n.g.	n.g.	n.g.	n.g.
Subtidal beach	<15 mm	19		0.84 (±0.05)	0.58 (±0.03)	0.20 (±0.03)	0.02 (±0.01)	n.g.	n.g.
Males	15-20 mm	6		0.13 (±0.06)	0.08 (±0.04)	n.g.	n.g.	n.g.	n.g.
	20-25 mm	5		0.06 (±0.06)	0.02 (±0.02)	n.g.	n.g.	n.g.	n.g.

between the smaller and larger size classes in June (Kruskal Wallis F=12.85, p=0.002), and July (Kruskal Wallis H=15.85, p<0.001). The smaller size classes <15 mm and 15-20 mm grew faster than the larger size class (20-25 mm). In August only whelks of <15 mm and 15-20 mm size classes increased in shell length and there was no significant difference between the two classes (F=1.23, p=0.299), whereas in September the <15 mm size class was growing faster than the 15-20 mm class (F=23.41, p=<0.001).

Female *Nassarius* originating from the subtidal beach increased in size during the warmer summer months (July to September), while growth ceased during the winter months (October to December). In particular, the growth rate of the <15 mm whelks was rapid between July and September, although there was a significant difference in the rate of shell growth between these three months (Kruskal Wallis: H=44.13, p<0.001), with fastest growth in July and slowest growth in September. Growth rate of the 15-20 mm and the 20-25 mm size classes was highest in July and August and there were no significant differences between the two months (for the 15-20 mm: Anova, F=0.55, p=0.476; for the 20-25 mm: Kruskal Wallis, H=0.37, p=0.543). Whelks <15 mm were still increasing in shell length up to October, while the growth rate of the 15-20 mm and 20-25 mm size whelks slowed down in September and August respectively. *Nassarius* >25 mm did not increase their shell length at any time. The monthly growth rate was significantly different between the smaller and larger size classes (July: Kruskal Wallis, H=33.19, p<0.001; August: Kruskal Wallis, H=33.39, p<0.001 and September: Kruskal Wallis, H=8.63, p=0.003). Small whelks (<15 mm) grew faster than the intermediate size class (15-20 mm), and larger whelks (20-25 mm) grew slower than all other size classes.

Male *Nassarius* from the subtidal beach increased in size during the warmer months between July and September, while growth ceased during the winter months (October to December). The growth rate of the <15 mm whelks was higher between July and September, although there was a significant difference between these three months (Kruskal Wallis: H=41.40, p<0.001), with fastest growth in July and slowest growth in September. Growth of the 15-20 mm and 20-25 mm whelks was highest in July and August and there were no significant differences between the two months (for 15-20 mm: Kruskal Wallis, H=0.37, p=0.546; for 20-25 mm: Kruskal Wallis, H=0.02, p=0.881). Although whelks of <15 mm were still growing at a slow rate up until

October, the 15-20 mm and 20-25 mm animals stopped growing in late August. No males >25 mm from the subtidal beach were used in the laboratory growth experiment. The monthly growth rate was significantly different between the smallest and largest size classes (July: Kruskal Wallis, H=20.76, $p<0.001$; August: Anova, F=58.01, $p<0.001$). The smallest whelks (<15 mm) grew faster than those of 15-20 mm and 20-25 mm size classes.

In conclusion, small *Nassarius reticulatus* of <15 mm and 15-20 mm grew faster than individuals of 20-25 mm size, while whelks >25 mm did not increase in shell length. Growth mainly occurred during the warm summer months between June and August, slowing down in September and October and ceasing completely during the colder winter months (November and December). Whelks of smaller size grew for longer compared to large whelks that terminated their shell growth earlier in the year.

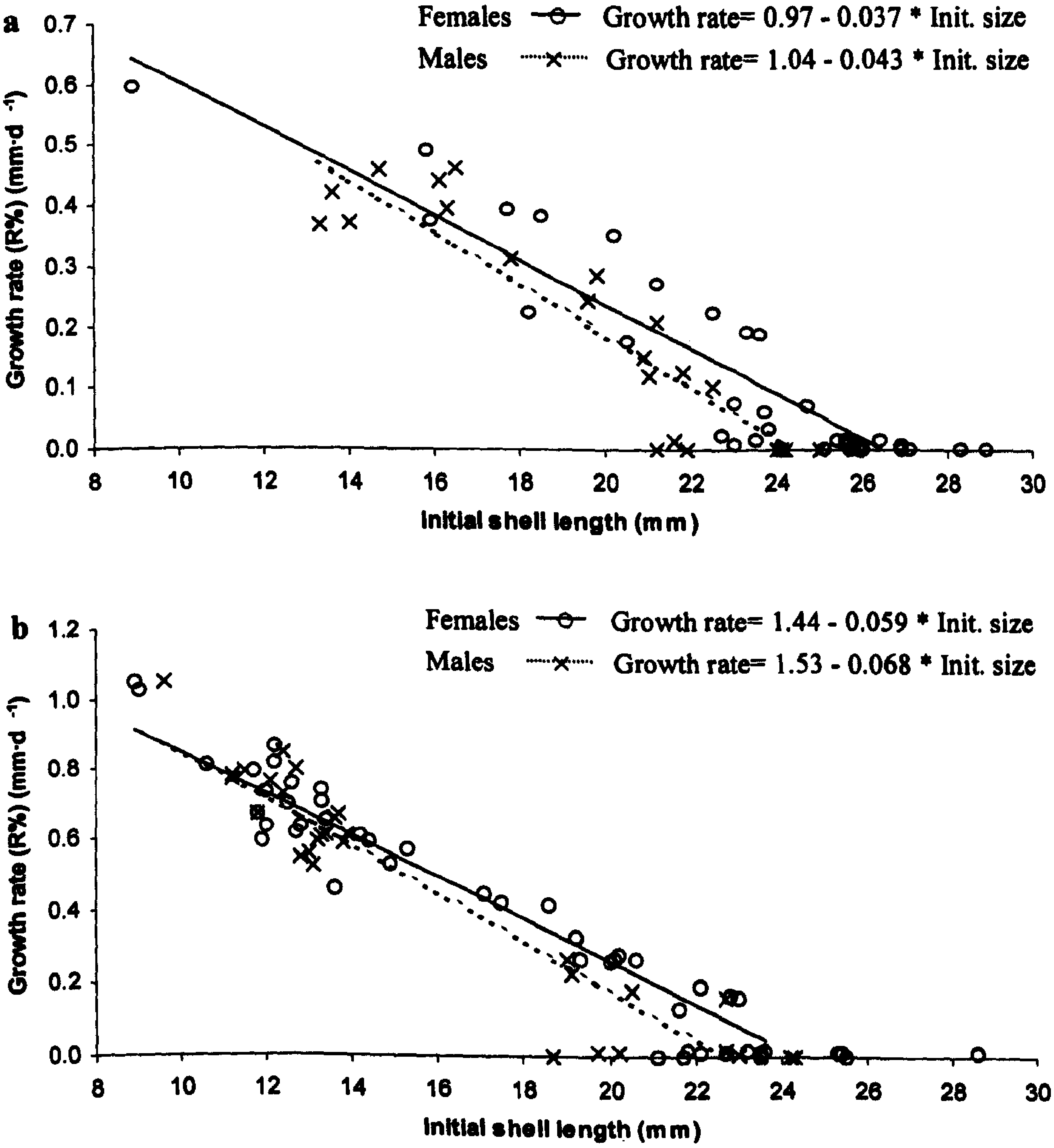
3.3.4.2 Comparison between the growth rate of male and female *Nassarius reticulatus* held in laboratory conditions

The comparison of the growth rate between female and male *Nassarius reticulatus* was conducted separately for whelks transferred from the intertidal lagoon (figure 3.12a) and the subtidal beach (figure 3.12b) to the laboratory, in order to eliminate any possible effect of origin on growth. A regression analysis between growth rate and initial shell length was used to eliminate the effect of size on growth rate. The growth rate was estimated for the period between July and August when all size classes grew faster.

A significant negative correlation was found between growth rate and initial shell length for females (Pearson's test $r= -0.920$, $p<0.001$) and males (Pearson's test $r= -0.918$, $p<0.001$) that originated from the intertidal lagoon. There was a significant linear regression between growth rate and initial shell length for females (F=170.57, $p<0.001$) and males (F= 113.03, $p<0.001$). In order to investigate possible differences in the relationship of growth rate and initial shell length between females and males from the intertidal lagoon a General Linear Model (GLM) analysis was performed. The residuals in all samples were normally distributed (females: $A^2= 0.531$, $p=0.162$; males: $A^2= 0.334$, $p=0.482$) and the data had equal variances (Bartlett's= 1.139, $p=0.723$). No significant difference was observed between males and females regarding the intercept

(F=0.46, p=0.502) or the slope (F=1.52, p=0.224) of the regression, indicating that shell growth is similar in female and male whelks from the intertidal lagoon. However, females attained a significantly larger mean shell length of 26.1 (±1.9) mm by the end of the experiment in December 2002, in comparison to males that had a mean shell size of 24.1 (±1.3) mm (Kruskal Wallis, H=21.54, p<0.001).

Figure 3.12 Relationship between growth rate (R%) (mm·d^{-1}) and initial shell length (mm) for females (circle symbol, solid line) and males (cross symbol, dash line) maintained in laboratory conditions that were collected from (a) the intertidal lagoon and (b) the subtidal beach.



A significant negative correlation was found between growth rate and initial shell length for females (Pearson's test r= -0.960, p<0.001) and males (Pearson's test r= -0.947,

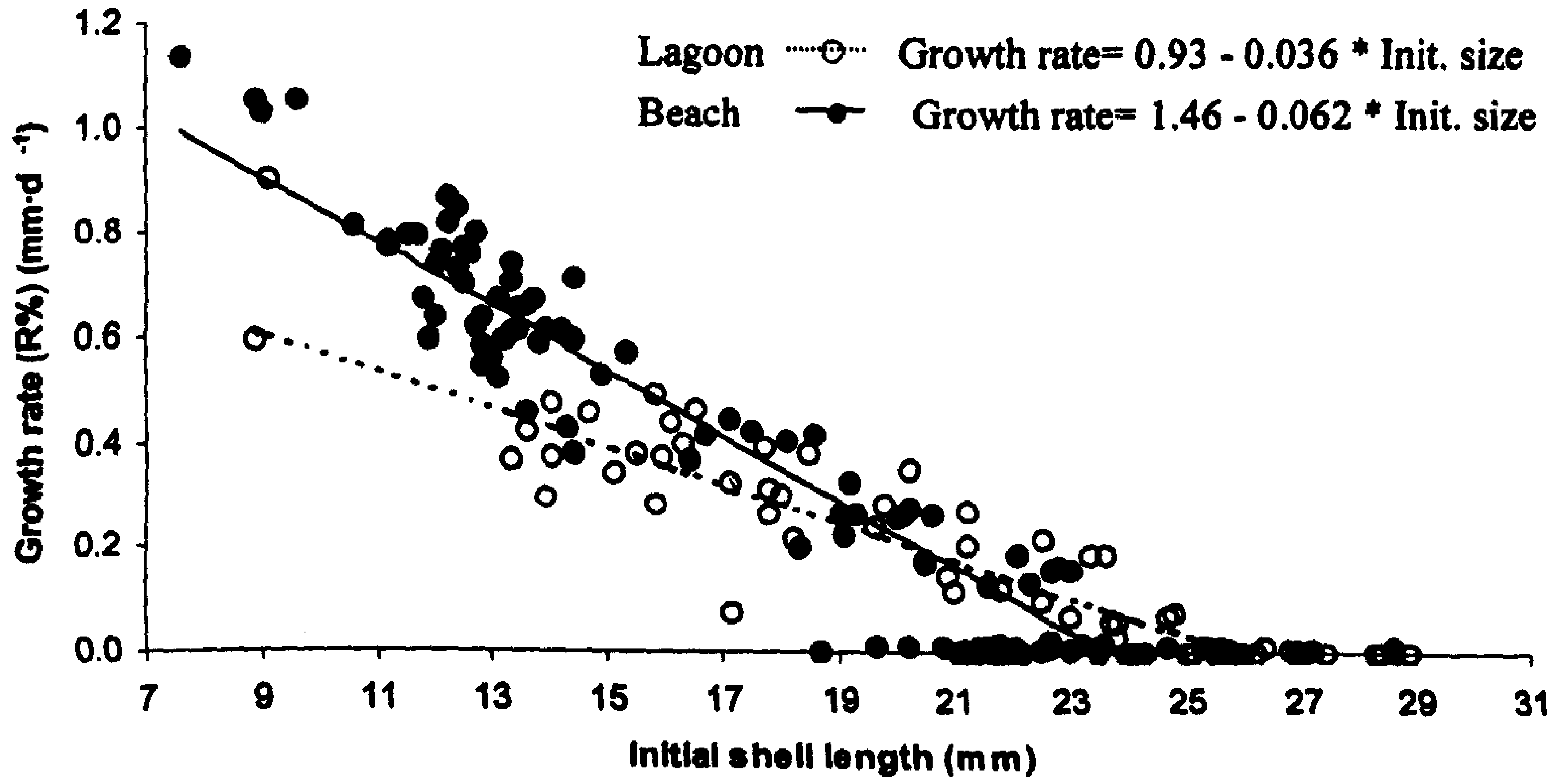
$p<0.001$) that originated from the subtidal beach. There was a significant linear regression between growth rate and initial shell length for females ($F=537.54$, $p<0.001$) and males ($F= 243.83$, $p<0.001$). In order to investigate possible differences in the relationship of growth rate and initial shell length between females and males from the subtidal beach, a GLM analysis was performed. The residuals in all samples were normally distributed (females: $A^2= 0.380$, $p=0.390$; males: $A^2= 0.414$, $p=0.317$) and the data had equal variances (Bartlett's= 1.325, $p=0.383$). No significant difference was observed between males and females regarding the intercept ($F=1.26$, $p=0.266$) or the slope ($F=3.15$, $p=0.080$) of the regression, indicating that shell growth is similar in female and male whelks from the subtidal beach. However, females attained a significantly larger mean shell length of 23.5 (±1.8) mm by December 2002, in comparison with males that had a mean shell size of 22.0 (±1.5) mm (Anova, $F=14.55$, $p<0.001$). In conclusion, there was no significant difference in growth of female and male *Nassarius reticulatus*, whether they originated from the intertidal lagoon or from the subtidal beach.

3.3.4.3 Comparison of the growth rate of *Nassarius reticulatus* held under laboratory conditions originating from the intertidal lagoon and the subtidal beach

The regression of growth rate with initial shell length was compared between whelks originating from the intertidal lagoon and the subtidal beach (figure 3.13). Having established that females and males have similar growth rates (section 3.3.4.2), whelks of both sexes were grouped together for further analysis. A significant negative correlation was found between growth rate and initial shell length of whelks from the intertidal lagoon (Pearson's test $r= -0.901$, $p<0.001$) and from the subtidal beach (Pearson's test $r= -0.947$, $p<0.001$). There was a significant linear regression between growth rate and initial shell length of whelks from the intertidal lagoon ($F=323.45$, $p<0.001$) and from the subtidal beach ($F= 831.82$, $p<0.001$). A GLM analysis was performed in order to investigate possible differences in the relationship of growth rate and initial shell length, between whelks originating from the intertidal lagoon and the subtidal beach. The residuals in all samples were normally distributed (lagoon: $A^2= 0.406$, $p=0.344$; beach: $A^2= 0.397$, $p=0.362$) and the data had equal variances (Bartlett's= 0.538, $p=0.067$). There was a significant difference in the intercept ($F=73.34$, $p<0.001$) and the slope

(F=72.75, p<0.001) of the regression between *Nassarius reticulatus* originating from the intertidal lagoon and the subtidal beach.

Figure 3.13 Relationship between growth rate (R%) (mm·d^{-1}) and initial shell length (mm) for *Nassarius reticulatus* maintained in laboratory conditions that originated from the intertidal lagoon (open circle and dotted line) and from the subtidal beach (closed circle and solid line).



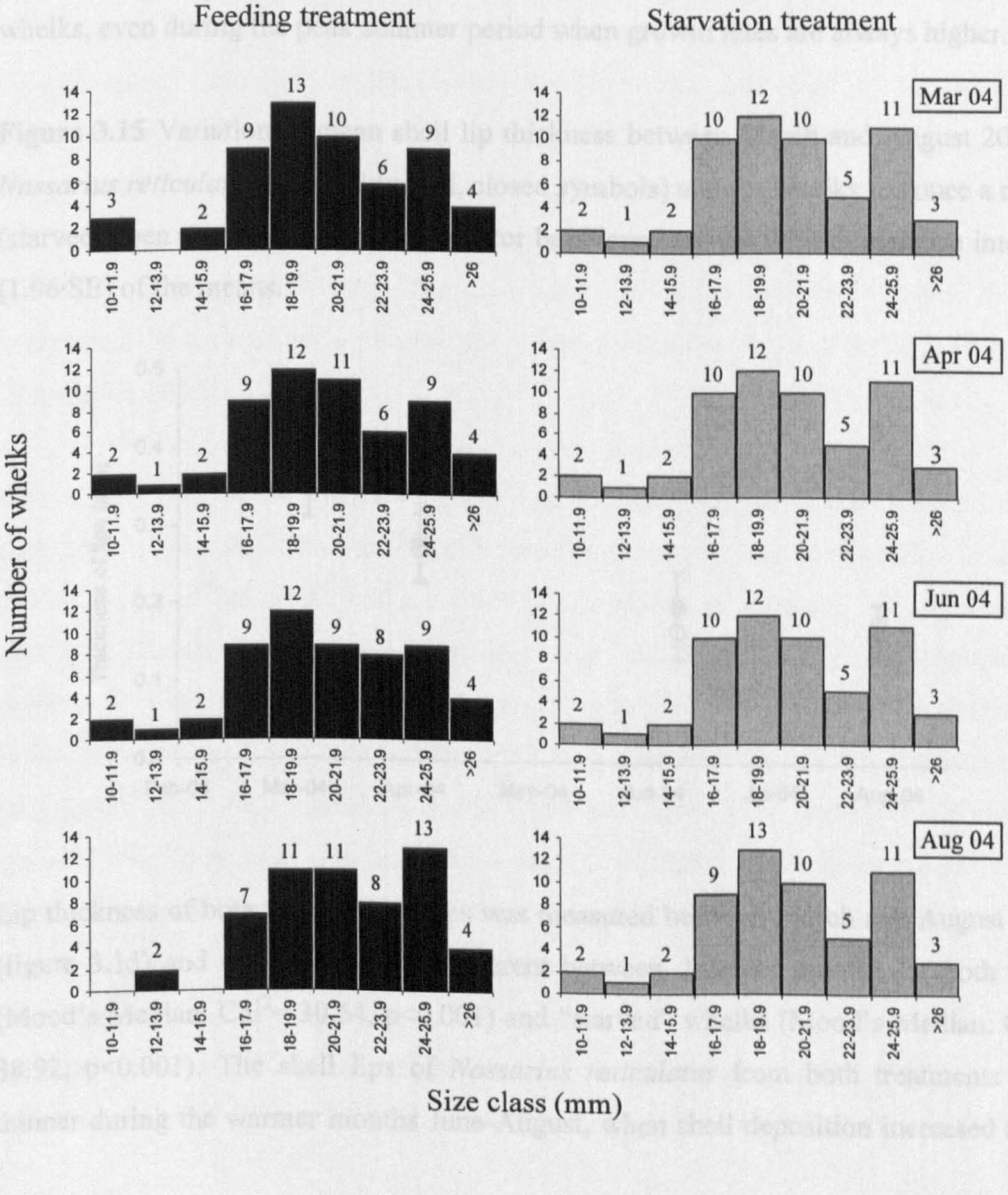
Although the *Nassarius reticulatus* were maintained under similar laboratory conditions a different growth profile characterised the whelks originating from the intertidal lagoon and the subtidal beach. The growth of small whelks (<21 mm) originating from the intertidal lagoon was lower than growth of small whelks from the subtidal beach. By contrast the growth rate of whelks >21 mm was higher for lagoon animals. Growth of the intertidal lagoon whelks ceased when the animals reached a maximum size of about 26 mm, while growth of the subtidal beach whelks stopped when the animals reached a maximum size of ~24 mm. However, there was considerable variability between individuals regarding the size at which growth ceased.

3.3.4.4 The effect of limited food supply on the growth of *Nassarius reticulatus* held under laboratory conditions

Of the 56 whelks held for 6 months on a limited food supply ("starvation": fed once a month) only 2 were dead at the end of the experiment in August 2004. Similarly 2

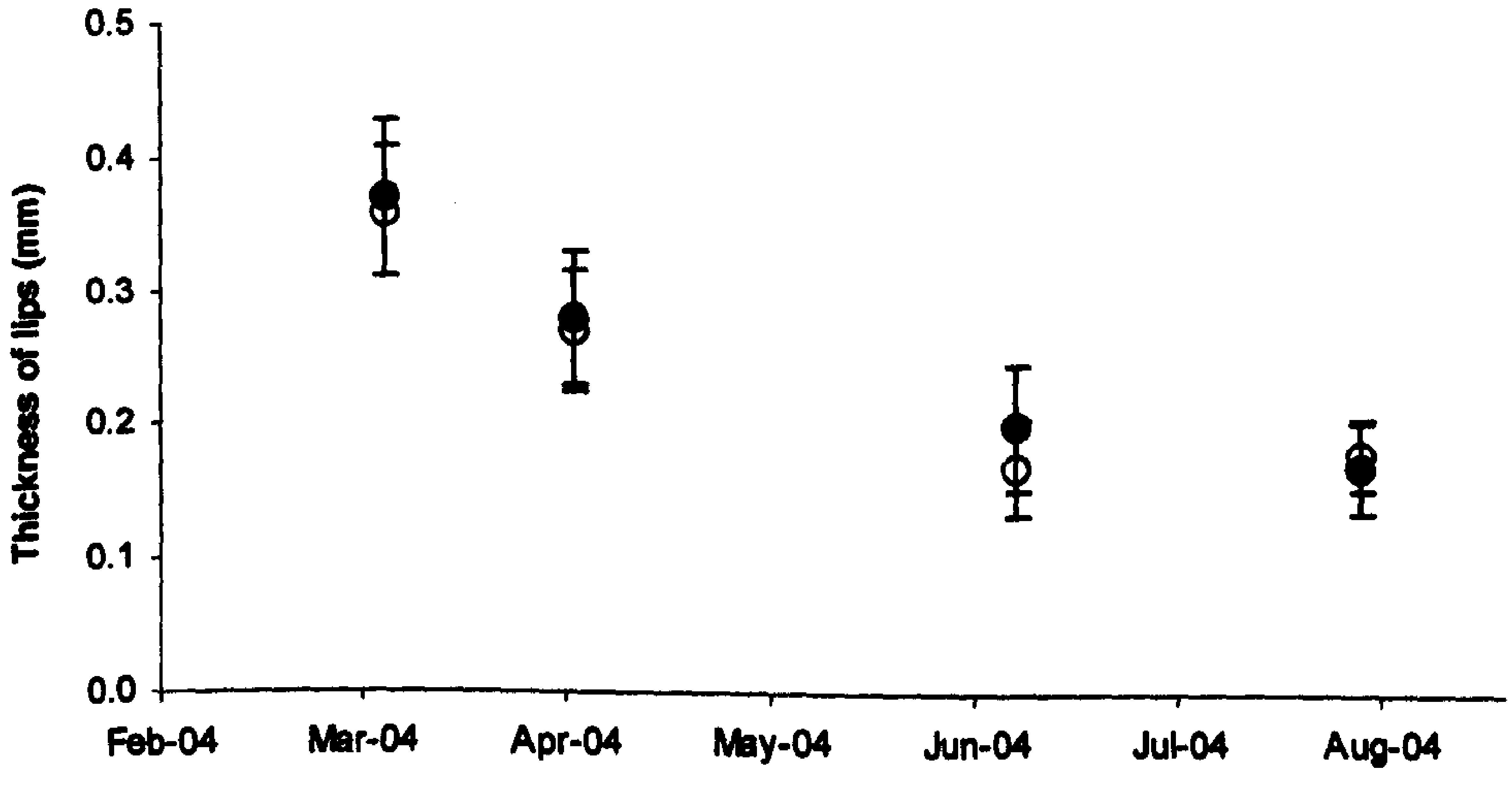
Nassarius reticulatus died in the "feeding" treatment (fed once a week). All four animals died between June and August. The whelks used in this experiment were not individually tagged, so it was not possible to follow the growth rate of each whelk in relation to its initial size. Instead the change in the composition of the length frequency distributions was followed between March and August 2004 for both the "feeding" and "starvation" treatments (figure 3.14).

Figure 3.14 Number of *Nassarius reticulatus* in different size classes (mm) for the "feeding" (black bars) and "starvation" (grey bars) treatments during the experimental period between March and August 2004. The number of individuals in each size class is shown above the bars. Samples were not measured in May and July.



In the "feeding" treatment the whelks increased in size as shown by the movement of more animals into the larger size classes. This change was not seen in the "starvation" group. Growth rate was higher during the period June to August than between March and April. By the end of August there were no whelks in the 10-11.9 mm and 14-15.9 mm size classes in the "feeding" treatment, and more individuals were part of the 22-23.9 mm and 24-25.9 mm size classes. By contrast in the "starvation" treatment no movement of the whelks to the higher size classes was observed. Only one individual grew from the 16-17.9 class to the 18-19.9 class between June and August. The reduced feeding regime ("starvation" treatment), during which food was provided only once a month, had no significant impact on the survival of *Nassarius reticulatus* compared with individuals that were fed once a week ("feeding" treatment). However, the growth rate of the "starved" whelks was significantly suppressed in comparison to the "fed" whelks, even during the peak summer period when growth rates are always higher.

Figure 3.15 Variation in mean shell lip thickness between March and August 2004 of *Nassarius reticulatus* fed weekly (fed, closed symbols) and for whelks fed once a month (starved, open symbols). The vertical error bars represent the 95% confidence intervals (1.96·SE) of the means.



Lip thickness of both treatment groups was measured between March and August 2004 (figure 3.15) and was significantly different between different months for both "fed" (Mood's Median: $Chi^2= 30.54$, $p<0.001$) and "starved" whelks (Mood's Median: $Chi^2= 38.92$, $p<0.001$). The shell lips of *Nassarius reticulatus* from both treatments were thinner during the warmer months June-August, when shell deposition increased at the edge of the shell (growing period). No significant difference in shell lip thickness between the "fed" and "starved" whelks was observed in any month between March and August (Kruskal-Wallis: for March H=0.01, p=0.965; for April H=0.01, p=0.914; for June H=0.43, p= 0.510; for August H=2.20, p=0.138). Although the low feeding regime reduced significantly the shell growth of the whelks in terms of shell length, nevertheless deposition of shell material at the shell margin still continued, albeit slowly.

3.3.5 Growth studies under field conditions determined from a mark-recapture experiment

Of the 165 tagged *Nassarius reticulatus* released into the intertidal lagoon in June 2002 (1st release), 14 were recaptured once and one individual was recaptured twice during the following 14 months (August 2003). From the second release of 101 tagged animals in September 2002, 27 whelks were recaptured once, 9 were recaptured twice, 4 were recaptured three times and one individual was recaptured four times in the following 24 months (September 2004). Finally, from the 300 tagged *Nassarius* released in May 2003 (3rd release), 95 were recaptured once, 17 were recaptured twice, 8 were recaptured three times and one was recaptured four times in the following 16 months (September 2004).

Marked and recaptured whelks were used to estimate the size of the intertidal lagoon population using "Jackson's positive method" (Jackson, 1939) as described by Begon (1979). Some of the conditions for using the Jackson's method are: a) a well defined sampling area which is visited regularly after the release of marked animals, b) recaptures in discrete time intervals (days, months etc.) which have to be roughly the same between subsequent samplings, and c) recaptures should be continued long enough in order to eliminate the effect of periodical (i.e. seasonal) activity and catchability of individuals. In the present study, three different releases took place in the intertidal lagoon but only the data from the third release were considered to be appropriate for the estimation of population size using "Jackson's positive method". Data from the first release (June 2002) were not considered appropriate because a number of marked gastropods were released in sites outside the main sampling area that were not visited regularly in the following months. Also data from the second release were not suitable because *Nassarius* released in September 2002 only began to be recaptured during the following spring. There was a low catchability during the winter, thus recapture data from the months following the release were not available. In both of the first and second releases a low number of marked animals was recaptured in the months following their release, resulting in an overestimation of the population size. The data from the third release (May 2003) were considered appropriate for analysis using Jackson's method because a) the area where the whelks were initially released was well defined and visited regularly every month, b) the release took place in May, thus a high recapture rate was expected during the following summer months, c) marked gastropods were recaptured regularly every month over a long period between June and December 2003 and d) a large number of whelks was initially marked and released in this trial, thus increasing the reliability of the method.

Data from the third release of marked and recaptured *Nassarius* and the calculations used to estimate the birth rate and population size are summarised in table 3.2. Details for the calculations are described in section 3.2.4. A monthly interval was used as the time interval. Using Jackson's positive method the estimated size of the *N. reticulatus* population in the intertidal lagoon in May 2003 was 3665 individuals with a constant birth rate of 0.108 (or 10.8%) per month. Bearing in mind that the sampling area of the intertidal lagoon is about 273 m^2, the estimated density of *N. reticulatus* is ~13.4 animals·m^{-2}. When the calculated standard error for ln(1-b) is antiloged a range of (1-b) and b values can be estimated, and when the standard error for $\ln q_0$ is antiloged a range of q_0 and N_0 values can be estimated. However, it has to be noted that the final standard errors are not symmetrical because they are produced after antiloging the estimated $SE_{\ln(1-b)}$ and $SE_{\ln q_0}$. Therefore the estimated range of the population size was between 3074 to 4370 individuals, with a birth rate of -0.066 to 0.254 per month. The calculation of birth rate gives only an estimated value which is subject to error, thus imperfect sets of data can produce "impossible" answers, such as a negative birth rate (see Begon, 1979). The negative estimation of birth rate included in the above range (-0.066) should be interpreted as a zero value, and not to be assumed as a positive loss.

One assumption in Jackson's positive method is that gain rate is constant, indicating that birth and immigration rates are not variable throughout the period between marking and recapture. This is applicable when the breeding and non-breeding periods are well defined and do not overlap within the period examined. However, in the current set of recapture data both the breeding period (late spring to summer) and non-breeding period (autumn to winter) are included, thus the birth rate of the lagoon population cannot be considered as constant. There are more advanced formulae of the Jackson's model that can include overlap of generations (birth and death) and movements into or out of the study area (immigration, emigration), but they require several marking and recapture occasions. In the present study only one applicable marking occasion took place (3rd release), thus the above ranges for birth rate and population size should only be used as estimates and not as definitive measurements.

Table 3.2. Summary of the Jackson's positive method results: number of marked *Nassarius reticulatus* initially released into the intertidal lagoon (r_i), the total number of gastropods (n_i) and the number of marked gastropods (m_i) that were collected during each monthly recapture (i), and the proportion of marked gastropods in each sample (q_i). The calculated birth rate (b) and the population size (N_O) are presented.

	May-03	Jun-03	Jul-03	Aug-03	Sep-03	Oct-03	Nov-03	Dec-03
i	0	1	2	3	4	5	6	7
r_i	300							
n_i		600	312	291	285	89	143	259
m_i		33	33	15	19	3	5	11
q_i		0.055	0.106	0.052	0.067	0.034	0.035	0.043
$\ln q_i$		-2.900	-2.247	-2.965	-2.708	-3.390	-3.353	-3.159

$\overline{\ln q}=-2.960$ $\bar{i}=4$

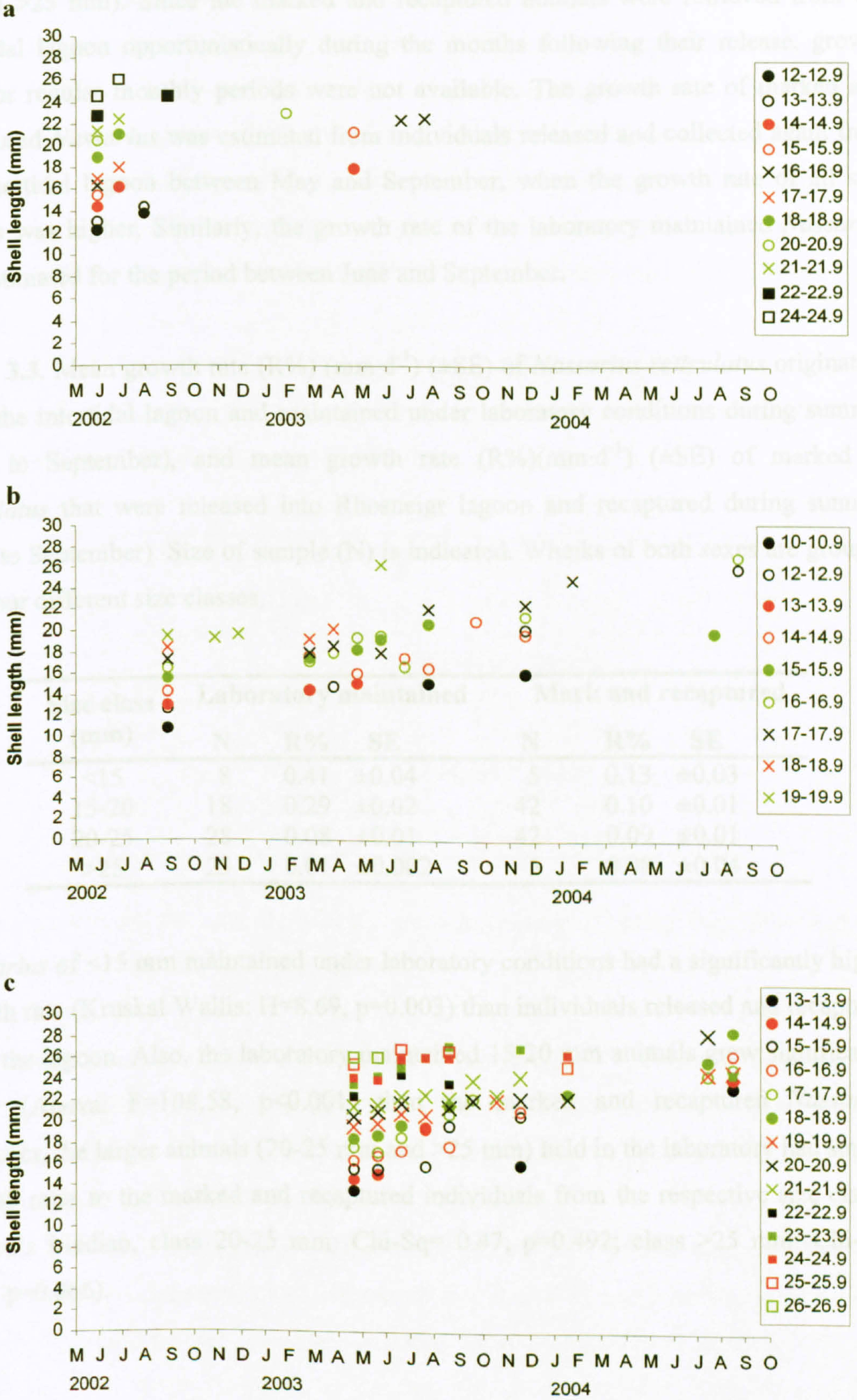
$\ln(1-b) = -0.114$ $SE_{\ln(1-b)} = 0.178$ $\mathbf{b = 0.108}$

$\ln q_0 = -2.503$ $SE_{\ln q_0} = 0.176$ $q_0 = 0.082$ $\mathbf{N_0 = 3664.9}$

The marked and recaptured whelks revealed information about the growth rate of *Nassarius reticulatus* in the intertidal lagoon. Whelks were grouped into size classes according to the shell size they had at the time of their initial release into the lagoon. The increase in mean shell length of each size class was estimated using the data from the *Nassarius* recaptured from the intertidal lagoon, and is shown in figure 3.16.

The growth rate of the recaptured *Nassarius* was compared with the growth rate of the laboratory maintained animals that were initially collected from the intertidal lagoon

Figure 3.16 Mean shell length (mm) of marked and recaptured *Nassarius reticulatus* from the first (a), second (b) and third (c) release into the intertidal lagoon. The whelks were grouped in size classes according to their initial shell length at the time of their release.



and were regularly fed (see table 3.3). As demonstrated previously (section 3.3.4.2) there was no significant difference in the growth rate of female and male *N. reticulatus*, thus the growth rate (R%) of marked-recaptured and laboratory maintained whelks was estimated after pooling together females and males into 4 size classes (<15, 15-20, 20-25 and >25 mm). Since the marked and recaptured animals were retrieved from the intertidal lagoon opportunistically during the months following their release, growth data for regular monthly periods were not available. The growth rate of marked and recaptured *Nassarius* was estimated from individuals released and collected again from the intertidal lagoon between May and September, when the growth rate of all size classes was higher. Similarly, the growth rate of the laboratory maintained *Nassarius* was estimated for the period between June and September.

Table 3.3. Mean growth rate (R%) ($mm \cdot d^{-1}$) (±SE) of *Nassarius reticulatus* originating from the intertidal lagoon and maintained under laboratory conditions during summer (June to September), and mean growth rate (R%)($mm \cdot d^{-1}$) (±SE) of marked *N. reticulatus* that were released into Rhosneigr lagoon and recaptured during summer (May to September). Size of sample (N) is indicated. Whelks of both sexes are grouped into four different size classes.

Size class (mm)	**Laboratory maintained**			**Mark and recaptured**		
	N	**R%**	**SE**	**N**	**R%**	**SE**
<15	8	0.41	±0.04	5	0.13	±0.03
15-20	18	0.29	±0.02	42	0.10	±0.01
20-25	28	0.08	±0.01	42	0.09	±0.01
>25	23	0.01	±0.002	5	0.08	±0.04

Nassarius of <15 mm maintained under laboratory conditions had a significantly higher growth rate (Kruskal Wallis: H=8.69, p=0.003) than individuals released and recaptured from the lagoon. Also, the laboratory maintained 15-20 mm animals grew significantly faster (Anova: F=108.58, p<0.001) than the marked and recaptured *Nassarius*. However, the larger animals (20-25 mm and >25 mm) held in the laboratory had similar growth rates to the marked and recaptured individuals from the respective size classes (Mood's Median, class 20-25 mm: Chi-Sq= 0.47, p=0.492; class >25 mm: Chi-Sq= 3.38, p=0.066).

3.3.6 Comparison of methods used to analyse the length-frequency distributions and a comparison with growth data from marked and recaptured *Nassarius reticulatus*

The length-frequency distributions were analysed using two different methods to describe the growth of *Nassarius reticulatus* at Rhosneigr: a) the Modal Progression Analysis (MPA) is a graphical method based on Bhattacharya (1967), which decomposes the population distribution into cohorts and follows the progressive growth of these cohorts with time and b) the LFDA method is a computerised method, which fits von Bertalanffy growth curves to length-frequency data. Both analytical methods resulted in estimation of the von Bertalanffy growth parameters (see table 3.4).

Table 3.4. Growth parameters of the von Bertalanffy growth equation for *Nassarius reticulatus* from the intertidal lagoon and the subtidal beach estimated using MPA and LFDA analysis of length-frequency distributions.

	Von Bertalanffy growth parameters				
	L_∞ (mm)	K	C	t_S	WP
		Intertidal lagoon			
MPA	28.6	0.70	0.78	-0.09	0.41
LFDA	27.5	1.16	1.00	0.08	0.58
		Subtidal beach			
MPA	25.5	0.85	1.00	-0.22	0.28
LFDA	26.8	0.84	0.66	-0.18	0.32

The estimations of the von Bertalanffy growth parameters using the MPA and the LFDA methods were not in full agreement:

- Estimations of the asymptotic maximum length (L_∞) were similar between the LFDA and MPA analysis. However, the LFDA analysis indicated a lower L_∞ than the MPA analysis for the intertidal lagoon, whereas for the subtidal beach the LFDA analysis indicated a higher L_∞ than the MPA analysis.
- In the intertidal lagoon the growth constant (K) from the LFDA analysis was more than double compared to the K estimated from the MPA analysis. The estimation of K for the subtidal beach animals was similar using both analytical methods.

- A strong seasonal variation in growth ($C>0.5$) was supported using both the MPA and LFDA methods. However, the LFDA analysis indicated that growth stops completely for a period of time in the intertidal lagoon ($C=1$), whilst the MPA analysis indicated that growth of *Nassarius* stops completely for a period of time on the subtidal beach.
- Analysis of the lagoon data by MPA analysis indicated that the period of slowest growth was at the end of September ($WP=0.41$), and growth started to increase again at the beginning of April ($t_S=-0.09$). The LFDA analysis of the lagoon animals indicated that the period of zero growth was shifted slightly later within the year, at the end of November ($WP=0.58$), and growth started to increase again after the end of May ($t_S=0.08$). Similarly, the MPA analysis for animals from the subtidal beach indicated that the period of zero growth was after the middle of August (WP= 0.28) and growth started to increase again after mid February ($t_S=-0.22$), while the LFDA analysis indicated that the period of reduced growth was slightly later within the year, at the beginning of September ($WP=0.32$), and growth started to increase again after the end of February ($t_S=-0.18$).

In order to evaluate which of the two methods was closer to reality, the growth patterns of *Nassarius reticulatus* in the lagoon described by the two length-frequency analytical methods, were compared with actual growth data from marked individuals that were released into the lagoon and recaptured opportunistically during the following months. The increase in shell length of marked and recaptured individuals during a roughly annual period (9 to 15 months) is summarised in table 3.5. A mark and recapture experiment was not conducted in the subtidal beach habitat, where the possibilities of recapture were dramatically decreased, because the sampling area was not as well constrained as the intertidal enclosed lagoon.

The growth lines fitted to the lagoon length-frequency data using the LFDA analysis, estimated that *Nassarius reticulatus* with a shell size of ~7-8.9 mm when they first entered the population, had reached a shell length of 15-16.9 mm after just 3 months, and they were already part of the 21-22.9 mm size class one year later. However, according to the actual growth increments measured in individuals released and recaptured from the lagoon, animals with a 10.8 mm shell length reached a size of 15.1 mm after one year and a size of about 21 mm after two years. The growth lines fitted by the LFDA analysis were described by a high growth constant ($K=1.16$), thus indicating that *N. reticulatus* can reach a size of 21-22.9 mm after just one year, whereas the marked and recapture *Nassarius* required at least 2 years to grow to that size. Growth of larger size classes as described by LFDA indicated that the 19-20.9 mm whelks reached a size of 25-26.9 mm one year later. Growth of the marked and recaptured animals with a size of about 21 mm reached a size of 24.8 to 28.6 mm one year later. The growth pattern of the larger size classes, indicated by the LFDA analysis was similar to the growth increments measured from the marked and recaptured animals. Although the LFDA analysis was quite efficient in estimating the growth of the larger size classes, growth of the smaller size classes was grossly over-estimated.

Table 3.5. Increase in mean shell length (mm) of different size classes of marked *Nassarius reticulatus* recaptured after a period of about one year (9 to 15 months) from the three releases into the Rhosneigr lagoon. The initial size class refers to the size classes that were presented in figure 3.16.

1st release - 5 June 2002				
Initial size class (mm)	**Date 1**	**Length 1**	**Date 2**	**Length 2**
14-14.9	June 02	14.5	May 03	18.1
15-15.9	June 02	15.5	May 03	21.4
16-16.9	June 02	16.3	July 03	22.5
2nd release - 30 September 2002				
10-10.9	Sept 02	10.8	Aug 03	15.1
12-12.9	Sept 02	12.8	Dec 03	20.2
14-14.9	Sept 02	14.3	Oct 03	20.9
15-15.9	Sept 02	15.5	Aug 03	20.7
16-16.9	Sept 02	16.6	Dec 03	21.4
17-17.9	Sept 02	17.4	Aug 03	22.1
3rd release - 21 May 2003				
13-13.9	Dec 03	16.1	Sept 04	23.3
14-14.9	Aug 03	19.5	Sept 04	24.1
16-16.9	Aug 03	19.3	Aug 04	24.5
18-18.9	July 03	19.6	Aug 04	25.7
17-17.9	Sept 03	20.8	Sept 04	25.1
19-19.9	Aug 03	20.6	Aug 04	24.7
18-18.9	Sept 03	21.6	Sept 04	28.6
19-19.9	Sept 03	21.4	Sept 04	24.8
20-20.9	Aug 03	22.6	Aug 04	28.2

The growth lines fitted to the intertidal lagoon length-frequency data using the MPA analysis, estimated that *Nassarius reticulatus* had a shell size of about 7-8.9 mm when they first entered the population and had reached a shell length of 15-16.9 mm after 7 months. According to measurements from *Nassarius* released and recaptured into the intertidal lagoon, whelks with a 10.8 mm shell length reached 15.1 mm after 11 months. Growth estimates using the MPA analysis were slightly higher than the observed growth increments obtained from the marked and recaptured whelks, however, they were more similar than the LFDA estimations. Furthermore, the growth increments for the larger size classes based on the MPA analysis were similar to the marked and recaptured growth increments. The MPA derived growth lines indicated that *N. reticulatus* of 15-16.9 mm reached a size of 19-20.9 mm in 7 months and a size of 23-24.9 mm in one year. Similarly, the 15.5 mm sized marked and recaptured whelks reached a size of 20.7-21.4 mm after 11 months and the 16.1-16.3 mm individuals reached a size of 22.5-23.3 mm in 9-13 months. The MPA derived growth lines indicated that gastropods of 19-20.9 mm reached a size of 23-24.9 mm after one year, while the marked and recaptured *Nassarius* of 19.3-19.6 mm size had attained a size of 24.1-25.7 mm after 12-13 months.

A more detailed comparison between the actual growth increments derived from linkage of cohort means in the MPA analysis (figure 3.8a) for the intertidal lagoon *Nassarius reticulatus*, with the growth increments derived from marked-recaptured data is presented in table 3.6. The cohorts growth increments from the MPA analysis were estimated, wherever possible, for periods of time similar to the periods covered by the marked and recaptured gastropod growth increments in table 3.5.

According to the MPA analysis of the length frequency data from the intertidal lagoon population, animals of 10 mm size reached 16.6 mm 10 months later, and individuals of 11.7 mm reached 20.6 mm 12 months later. Respectively, the mean shell length of marked and recaptured whelks increased from 10.8 to 15.1 mm after 11 months, and from 12.8 to 20.2 mm after 15 months. In addition, similar growth increments were estimated from the MPA analysis for the 14.9 mm sized *Nassarius* that reached a shell length of 21.0 mm after one year, and from the marked-recaptured gastropods that increased from 14.3 to 20.9 mm after 13 months. Whelks of 15.0-17.9 mm size in the MPA analysis reached a size of 22.8-23.8 mm after 10-12 months, whereas marked and recaptured individuals of a similar size-range (15.5-17.4 mm) reached a size of 20.7-23.3 mm after 9-15 months. Whelks of ~20.0 mm in the MPA analysis were 25.4 mm 13 months later, and the marked and recaptured whelks of 19.6-20.8 mm were also close to 25 mm 12-13 months later.

Table 3.6. Comparison between growth increments of cohorts of *Nassarius reticulatus* estimated from the MPA intertidal lagoon population analysis, and growth increments as observed in marked gastropods that were released and recaptured opportunistically from the intertidal lagoon. The shell measurements are mean values from individuals that belong to the same size class.

Size class (mm)	**MPA increments for cohorts (lagoon)**				**Marked and recaptured *Nassarius***			
10-10.9	Sept 02	10.0	July 03	16.6	Sept 02	10.8	Aug 03	15.1
11-12.9	June 02	11.7	June 03	20.6	Sept 02	12.8	Dec 03	20.2
14-14.9	Sept 02	14.9	Sept 03	21.0	Sept 02	14.3	Oct 03	20.9
15-16.9	Dec 02	15.0	Dec 03	22.8	June 02	15.5	May 03	21.4
	Apr 02	15.3	Apr 03	23.8	Sept 02	15.5	Aug 03	20.7
					June 02	16.3	July 03	22.5
					Sept 02	16.6	Dec 03	21.4
					Dec 03	16.1	Sept 04	23.3
17-17.9	Feb 03	17.9	Dec 03	22.8	Sept 02	17.4	Aug 03	22.1
19-20.9	Aug 02	20.0	Sept 03	25.4	July 03	19.6	Aug 04	25.7
					Sept 03	20.8	Sept 04	25.1
21-23.9	Apr 02	22.8	Mar 03	24.6	Sept 03	21.4	Sept 04	24.8
	Sept 02	23.9	Sept 03	25.4	Sept 03	21.6	Sept 04	28.6
					Aug 03	22.6	Aug 04	28.2

The growth increments of the marked and recaptured animals in the intertidal lagoon were used in order to calculate the von Bertalanffy growth parameters using Munro's method (1982). Multiple recaptures were treated as independent measurements, and a growth increment was calculated for each period between successive recaptures. Again the growth parameters estimated from the marked and recaptured whelks were close to the MPA estimations. The L_∞ estimated from the mark-recapture data was 28.6 and this value was equal to the one calculated from the MPA analysis of the size-frequency distribution. The growth constant value (K) from the mark-recapture data was 0.93, relatively higher than the MPA estimated value (0.70).

3.4 DISCUSSION

The catchability of *Nassarius reticulatus* in the intertidal lagoon in Rhosneigr was significantly higher (124-150 *Nas*/trap) during the summer months from June to August 2002 and 2003, when the surface seawater temperature was also at its highest (18-22°C). Lower numbers of *N. reticulatus* (29.7-64.8 *Nas*/trap) were collected per trap during the winter months in 2002 and 2003 when the temperature was below 11.8°C. A similar seasonal pattern regarding the catchability of *N. reticulatus* was observed in the subtidal beach in Rhosneigr. More whelks (13.6-14.9 *Nas*/trap) were collected during July 2002 and 2003 at temperatures >15.9°C and fewer (≤5 *Nas*/trap) during the winter and early spring months when the temperature fell below 9.5°C. Tallmark (1980) found that the abundance of *N. reticulatus* in Kvarnbukten Bay (Gullmar Fjord, Sweden) was higher during the summer months. Reduced activity due to low temperatures can be a factor resulting in lower catchability during the winter. *N. reticulatus* were immobile during the winter in Kvarnbukten Bay when the water temperature was below 4°C (Tallmark, 1980). Activity resumed when the water temperature rose above 5-7°C and mobility was increased during the summer months (Tallmark, 1980). Morton & Chan (2004) also reported that the catchability of *N. festivus* in Hong Kong was higher during summer because activity was increased at the higher temperatures.

More *Nassarius reticulatus* were collected from the intertidal lagoon within 30 minutes, compared to the subtidal beach, thus it can be concluded that the intertidal lagoon supports a significantly larger population of *N. reticulatus* than the subtidal beach. The percentage organic content in the sediment in the intertidal lagoon was 1.8 times higher than in the subtidal beach sediment, thus indicating higher availability of organic material. Tallmark (1980) found that the population density of *N. reticulatus* in Kvarnbukten Bay (Gullmar Fjord, Sweden) was positively correlated with the organic content of the sediment. Similarly, the catchability of *N. festivus* was higher on a Hong Kong beach where food availability was greater due to dumping of by-catch by artisanal fisheries (Morton & Chan, 2004). Large amounts of carrion can be trapped for a longer time in a sheltered pool surrounded by rocks, such as the Rhosneigr lagoon habitat, whilst on a subtidal wave-exposed shore the available carrion can be easily carried away by currents. In addition the higher seawater temperatures that were recorded in the intertidal lagoon during the summer months could result in enrichment of the lagoon population by higher reproductive activity and recruitment. The density of recruits of *N. pauperatus* was higher on a sandflat in Gulf St.Vincent (South Australia), where extensive water pools were formed during low tide, because larvae could be entrapped in the pools where the higher seawater temperatures enhanced the development and increased the survival of recruits (McKillup *et al.*, 1993). Tallmark (1980) suggested that irregular settlement of larvae could be responsible for variations in population size between different sites.

The limits of a population are easier to recognise where animals live in ponds surrounded by rocks, so in these cases the whole population can be sampled and mark-recapture methods truly measure population size (Begon, 1979). The size of the intertidal lagoon population, estimated using Jackson's positive method from the 3rd marked-recaptured experiment, was 3665 animals in May 2003 (range from 3074 to 4370) for a sampling area of 273 m^2, resulting in a density of 13.4 $animals.m^{-2}$. Tallmark (1980) reported a range of densities from 4.8 to 18 *N. reticulatus*·m^{-2} at different stations in Kvarnbukten Bay (Gullmar Fjord, Sweden), while much higher densities of 40-60 animals·m^{-2} were estimated in the newly colonised lake Grevelingen (Netherlands) (Lambeck, 1984). The monthly birth rate of *N. reticulatus* into the Rhosneigr intertidal lagoon population was estimated to be 10.8% and it was assumed to be constant. However, bearing in mind that the time between the initial release (May 2003) and the final recapture (December 2003) of the marked whelks included both breeding (summer) and non-breeding (autumn, winter) periods, it can be concluded that the birth rate was not constant. The more advanced formulae of the Jackson's model that can process mark-recapture data with variable gain and loss rates require several marking and recapture occasions. However, in the present study only one marking occasion took place. The estimated population size from this experiment provided a rough indication of the population extent in the intertidal lagoon.

The distribution of *Nassarius reticulatus* in the intertidal lagoon and on the subtidal beach were described by multimodal length-frequency histograms. The intertidal lagoon population included whelks with a range of sizes from 7-8.9 mm to 29-33.2 mm, whilst the range of individuals from the subtidal beach was from 5-6.9 mm to 29-30.9 mm. The smallest *N. reticulatus* collected from the intertidal lagoon were 7-8.9 mm and they were present in the population mainly from February to April (2.1-5.1% of the total population). Low numbers of similar small-sized whelks were also collected during the summer and early autumn months. The smallest sizes collected from the subtidal beach were 5-6.9 in 2002, although in 2003 the smallest ones collected were 7-8.9 mm. Small individuals were collected from the subtidal beach in almost all of the monthly samples. The highest numbers of small whelks in the subtidal population were collected in May (6.78%), August (6.26%) and October (7.38%) during the year 2002, and in February (6.90%), March (4.92%) and May (8.05%) of 2003. Barroso & Moreira (1998) also noted a lack of sizes smaller than 6.5 mm in their samples from the Ria de Aveiro, and suggested that there may be nursery sites for settlement from where the gastropods migrate into the main population when they are almost one year old.

According to the growth curves fitted to the length-frequency data using the MPA analysis, *Nassarius reticulatus* larvae hatched and settled during the spring and summer, and appeared as part of the population between February and April the next year at a size of 7-8.9 mm. A similar pattern of annual recruitment was observed for both the intertidal lagoon and the subtidal beach populations. Lambeck (1984) reported that *N. reticulatus* juveniles appeared in the population of the newly colonised saline lake Grevelingen (Netherlands) in August and that one generation was produced per year. Also Barroso *et al.* (2005a) indicated a single annual recruitment for *N. reticulatus* in the Ria de Aveiro (Portugal), where larvae hatched in early spring, settled in late spring and were part of the population at a size of 5 mm by September of the same year. Tallmark (1980) indicated that *N. reticulatus* larvae settled in the deeper area of Lysekil harbour and only sexually mature individuals (3 years old) moved into the more shallow area of Kvarnbukten Bay. However, recruitment in Kvarnbukten Bay was not annual, and was observed only three times during the 5 year study period (Tallmark, 1980).

The rate of natural mortality showed a slight seasonal pattern with relatively higher values during the spring and summer months. The higher seawater temperatures and the greater reproductive effort may be responsible for the decreased survival during the summer period. Spight (1982) demonstrated a marked seasonal variability in mortality rates of *Thais emarginata* and *T. lamellosa* in San Juan Island (Washington), indicating that most individuals died during summer when the environmental conditions were more stressful. Although the seasonal fluctuations in mortality rate were similar in the Rhosneigr intertidal lagoon and the subtidal beach habitats, a higher annual rate of natural mortality was estimated in the intertidal lagoon population (0.778) compared with the subtidal beach population (0.577). The intertidal lagoon is shallower compared to the subtidal area, and both depths become even more reduced during low tide, especially during spring tides. Thus in the intertidal lagoon higher seawater temperatures are developed during summer and lower temperatures during winter. Animals in the intertidal lagoon can experience temperatures up to 22°C during the summer months and down to 5.9°C during winter, while individuals in the subtidal area experience highest temperatures of 17.1°C in summer and lowest temperatures of 6.6°C during winter. In addition, predation by crabs is higher in the intertidal lagoon, because it is more sheltered than the subtidal beach. The higher summer and lower winter temperatures, as well as predation pressure in the intertidal lagoon, could be responsible for the lower % survival rate per annum in the lagoon environment (45.9%) than on the subtidal beach (56.2%).

The von Bertalanffy parameters for the intertidal lagoon and subtidal beach populations of *Nassarius reticulatus* were estimated from the analysis of length-frequency distributions using a graphical method (MPA) and a computerised method (LFDA). The results, however, obtained from the two methods were not in agreement. The validity of the results of both methods was examined by comparison with growth data derived from a marking-recapture experiment, as proposed by Grant *et al.* (1987). The growth pattern of *N. reticulatus* as indicated by the graphical method MPA was closer to the measured growth of marked and recaptured individuals. Computer programs, such as the LFDA analysis, based on mathematical procedures that are able to fit growth curves to size-frequency data are often designed for fish populations in which the movement of individuals will average the impact of environmental variations (Grant *et al.*, 1987). Such methods may not therefore be appropriate for relatively non-mobile invertebrate populations where large variations of recruitment can occur from year to year or where growth rates may fluctuate with time due to the effect of population density on growth (Grant *et al.*, 1987).

Having established that the MPA analysis of the length-frequency distributions was more similar to the mark-recapture data, the comparison of growth parameters between the intertidal lagoon and the subtidal beach was based on the interpretation of the MPA analysis. Whelks from the intertidal lagoon had a higher maximum asymptotic length and a lower growth constant (L_∞=28.6 mm, K=0.70) than those from the subtidal beach (L_∞=25.5 mm, K=0.85). Lambeck (1984) noted that growth rates can be lower in *Nassarius reticulatus* populations with higher densities, such as the intertidal lagoon in the present study, due to increased intra-specific competition. A strong seasonality to the growth curve was observed for both populations, however, those in the subtidal area stopped growing for a period of time. Although the subtidal beach population was characterised by a higher growth constant, a lower asymptotic length was reached finally compared with the intertidal lagoon animals, because growth in the subtidal beach ceased completely for a period of time after mid August. Animals in the intertidal lagoon were still growing during August, when the seawater temperature was 19.0-

20.1°C, and their growth decreased after the end of September (*WP*=0.41) when the temperature dropped to 15.9-16.4°C. Growth of *Nassarius* in the subtidal area ceased more than a month earlier, after mid August (*WP*=0.28), because the seawater temperature had already dropped to 16.0-17.1°C. In the intertidal lagoon growth of *Nassarius* began to increase again at the beginning of April (t_S=-0.09), when the seawater temperature increased to 13°C. However, in the subtidal beach growth started to increase more than a month earlier, after mid February (t_S=-0.22), although the temperature was just 6.6-8.5°C. Maybe the reason for the delayed growth stimulation in the intertidal lagoon was due to the fact that animals in the lagoon experienced temperatures <7.1°C for a 3-month period during winter, while the temperature in the subtidal beach dropped below 8.5°C only in February (6.6°C).

The MPA fitted growth curves of *Nassarius reticulatus* during the present study indicated that in the intertidal lagoon the larvae hatched and settled during summer, and the new recruits appeared in the population at a size of 7-8.9 mm from February to April the following year. *Nassarius* reached a size of 15-16.9 mm by the end of September, one year after their settlement, and a size of 23-24.9 mm, 25-26.9 mm and 27-28.9 mm in September, two, three and four years respectively after their settlement. In the subtidal beach however, although the new recruits of 7-8.9 mm (February-April) had also reached a size of 15-16.9 mm by the end of September one year after settlement, a smaller size of 21-22.9 mm, 23-24.9 mm and 25-26.9 mm was reached by September two, three and four years respectively after settlement. Unfortunately, growth studies of *N. reticulatus* in the existing literature have only reported the increase in shell length during time in different size classes and there are no available estimations of absolute growth rate values, such as the von Bertalanffy growth constant parameter (*K*) (Ekaratne & Crisp, 1984). Thus, only a broad comparison of the present study with older studies can be attempted, in terms of size range achieved at the respective age classes.

Tallmark (1980) indicated that the average shell length of *Nassarius reticulatus* in Kvarnbukten Bay (Gullmar Fjord, Sweden) was 5.5, 11 and 16.5 mm for the first three year classes respectively. The shell length of *N. reticulatus* in lake Grevelingen (Netherlands) was 6.8 mm after one growing season and about 15-16 mm after the second growing period (Lambeck, 1984). Barroso *et al.* (2005a) indicated that the length of *N. reticulatus* in the Ria de Aveiro (Portugal) was 6.7-7.8 mm after the first year, 12.1-14.5 mm after two years, 18.0-19.5 mm after 3 years, 22.7-23.6 mm after four years and 26.1-26.9 mm after five or more years. During the present study in Rhosneigr, whelks had a fast growth rate and reached a size of 15-16.9 mm after two growing seasons, similarly with *Nassarius* in the lake Grevelingen. However, Tallmark (1980) and Barroso *et al.* (2005a) found a lower growth rate, thus whelks of similar age were only 11 mm in Kvarnbukten Bay and 12.1-14.5 mm in Ria de Aveiro, respectively.

The results of the laboratory growth experiments agree with the conclusions of the length-frequency analysis of the intertidal and subtidal populations, and similarly indicate a strong seasonal growth pattern. A significant seasonal variation in growth rate was observed for both female and male *Nassarius reticulatus* that were collected from the intertidal lagoon or the subtidal beach and maintained under laboratory conditions. The growth rate of whelks was highest during the summer months, lower during autumn, and zero during the winter months. Seasonal variations in the growth rate of Nassariidae species have been reported in several previous studies. Scheltema (1964) reported that growth of *N. obsoletus* in the Great Pond area (Falmouth, Massachusetts) occurred mostly during the summer months, while during late autumn, winter and early spring, there was essentially no growth. The growth rate of *N. reticulatus* in lake Grevelingen (SW Netherlands) started to resume between mid-April and early-June, when the temperature was at about 14°C, and increased during the summer months (Lambeck, 1984). A seasonal pattern of shell growth was also observed for *N. reticulatus* in the Ria de Aveiro (Portugal) with minimum growth during winter and maximum growth during summer (Barroso *et al.*, 2005a). The laboratory growth experiments and the length-frequency analysis of a *N. festivus* population in Hong Kong conducted by Morton & Chan (2004) also indicated a seasonal variation in growth rate. Increased growth rates were observed when the temperature was higher, during summer and late autumn, and decreased or negligible growth rates were observed during winter and spring when the temperature was lowest (Morton & Chan, 2004).

Studies of other gastropod species such as *Nucella lapillus*, *Littorina littorea* (Ekaratne & Crisp, 1984) and *L. nigrolineata* (Hughes, 1980), which have been conducted in the same area as the present study (Anglesey, North Wales), indicated that growth rates were minimal during the winter months (December-March) and maximal during summer (June-September). Ekaratne & Crisp (1984) found that the acceleration of growth during early spring was slow, while deceleration during autumn was relatively abrupt. Decrease of *Nassarius obsoletus* growth rates has been related to the cessation of feeding activity during winter, due to the lower temperatures and to the decreased availability of food (Scheltema, 1964). Edwards & Huebner (1977) reported that *Polinices duplicatus* were inactive for four months during winter in Barnstable Harbour (Massachusetts), and during that period they remained quiescent in the sand without withdrawing into their shells.

The von Bertalanffy growth curves that were fitted to the length-frequency data of the intertidal and subtidal populations indicated that smaller animals grew faster. Accordingly, the laboratory experiment indicated that growth rate was size dependent for both female and male *Nassarius reticulatus* originating from the intertidal lagoon or the subtidal beach and maintained under laboratory conditions. Whelks with a smaller initial size (<15, 15-20 mm) grew faster than large whelks (20-25 mm), while whelks of the biggest size class (>25 mm) did not grow at all during the whole experimental period. In addition, according to their size class, individuals were growing for a different length of time. Whelks of the <15 mm size class were still growing in September and October, whilst those of 15-20 mm and 20-25 mm size classes stopped growing earlier, at the end of summer. The period of gonad development for *N. reticulatus* occurs between January and February (Barroso & Moreira, 1998), and the breeding and egg laying period occurs from the end of spring until late summer (Lebour, 1937; Barnett *et al.*, 1980; Tallmark, 1980; Fretter & Graham, 1984; Lambeck, 1984; Barroso & Moreira, 1998). When *N. reticulatus* becomes sexually mature energy is allocated to reproduction at the expense of shell growth. Thus the growth rate of whelks in the older size classes (>25 mm) was zero, even during the summer months.

Shell growth of *Nassarius* species decreases abruptly after the whelks become sexually mature, probably due to a strategic shift of energy allocation from growth to reproduction (Scheltema, 1964; Tallmark, 1980). Tallmark (1980) reported that the growth of *N. reticulatus* in Gullmar Fjord (Sweden) was fast during the first 3 years with a length increment of about 5.5 mm per year, but after the 4th year, when snails became sexually mature, growth rate decreased and became irregular. Most of the snails (70-80 %) that were larger than 20 mm did not grow at all in some years (Tallmark, 1980). Similarly, in the Ria de Aveiro (Portugal) *N. reticulatus* grows for the first five years of life and only a small increment of shell material is added during subsequent years (Barroso *et al.*, 2005a). Bryan *et al.* (1993) reported that the shell length of *N.*

reticulatus in the Yealm estuary (SW England) increases over the first 6 years of life and then tends to decline, probably because the very low annual growth rate does not compensate for severe erosion of the shell margin. The growth rate of juvenile *N. festivus* was higher in comparison to the growth rate of adults, due to the fact that small individuals invest more energy in growth, while larger individuals allocate more energy into marginal growth and reproduction (Morton & Chan, 2004).

No significant difference was found in the present study between the growth of male and female *Nassarius reticulatus* that originated from the intertidal lagoon or the subtidal beach. Similar growth rates between males and females have also been reported for other gastropod species such as *Ilyanassa obsoleta* (Curtis, 1995), *Polinices duplicatus* (Edwards & Huebner, 1977) and *Concholepas concholepas* (Rabi & Maravi, 1991). Although growth rate was not different between female and male *N. reticulatus*, females attained a significantly larger length than males by the end of the experimental period. Barroso *et al.* (2005a) demonstrated that female *N. reticulatus* attained a significantly larger size than males in Portugal. Considering that females grow at the same rate as males but achieve a larger size it can be concluded that females have a greater longevity.

The growth patterns of *N. reticulatus* were not similar between the intertidal lagoon and the subtidal beach populations as was previously indicated by the length-frequency analysis. In addition, a significant difference was found concerning the growth rate of individuals that were originally collected from the intertidal lagoon and the subtidal beach, even though all the animals were maintained under the same laboratory conditions for a period of ~ 6-7 months. The growth rate of animals <21 mm in the laboratory was lower for intertidal lagoon animals, however, the growth rate of whelks >21 mm was higher for the lagoon originated animals. The length-frequency analysis also indicated that the larger size classes grew faster in the intertidal lagoon. Growth in the laboratory of the intertidal lagoon *Nassarius* ceased when the animals reached a maximum size of about 26 mm, while growth of the subtidal beach animals stopped at a maximum size of about 24 mm. The length-frequency analysis also indicated a higher L_∞ for animals from the intertidal lagoon. All size classes that originated from the subtidal beach stopped growing in the laboratory about a month earlier than the ones originating from the intertidal lagoon. Thus, the smaller size classes of <15 and 15-20 mm of the intertidal lagoon originated whelks were growing until October and

September respectively, whilst animals originating from the subtidal beach stopped growing after September and August respectively. Similarly, the length-frequency analysis indicated that growth in the intertidal lagoon decreased after September, while growth of subtidal beach animals decreased a month earlier, after the end of August.

The above observations indicate that growth patterns of *Nassarius reticulatus* are undoubtedly different between individuals in the intertidal lagoon and the subtidal beach, and in addition, the different growth patterns are maintained even if the animals are grown under similar controlled conditions. Although *N. reticulatus* from the lagoon grow slower during the first stages of life, they grow for a longer period within the year, and they retain a higher growth rate when their size shifts to a bigger size class, thus cessation of growth after sexual maturity occurs when they have already achieved a bigger size. Morton & Chan (2004), also observed that the growth rate of *N. festivus* was significantly different between 3 different ecosystems, and indicated that the same differences in growth rate were maintained when whelks from the three sites were held under the same laboratory conditions.

During the present study the survival of *Nassarius reticulatus* was not significantly reduced in individuals that were maintained under a limited feeding regime in the laboratory. However, whelks that were provided with food regularly showed a considerable amount of shell length growth especially during the summer months, while the limited fed *Nassarius* did not grow at all. Tallmark (1980) reported that survival of *N. reticulatus* that were maintained under a complete starvation treatment for 5 months in the laboratory was 65%, while a higher percentage of gastropods (95%) survived under a regular feeding regime. The growth rate of starved whelks was zero (Tallmark, 1980). Only the smallest size classes of starved whelks that were provided with non-sterile sand (non-detritus free), collected from the field and renewed every week, increased in shell length, but even under these conditions growth was about 50% less in comparison to fed whelks held under the same conditions (Tallmark, 1980).

Measurement of shell lip thickness of regularly fed whelks indicated that *Nassarius* forms thinner lips during the warmer months from June to August. During periods of increased shell growth new material was constantly deposited on the edge of the shell, resulting in thinner lips. During winter shell growth was reduced and the edge of the shell lips was thickened. Tallmark (1980) reported that the shell of *N. reticulatus* increased only when the water temperature exceeded 14°C, from early June to late August, and that the new shell was very thin. During autumn and winter the new part of the shell was thickened (Tallmark, 1980). Barroso *et al.* (2005a) indicated that smaller *N. reticulatus* developed a thick shell lip (1 mm) with teeth during the winter, while in summer the lips became very thin (0.1-0.2 mm) and the teeth disappeared. In larger individuals the shell lip gradually thickened and when they reached a size of about 26 mm, the lip became permanently white and thick (1.8 mm) with a ridge of elongated teeth along the internal surface (Barroso *et al.*, 2005a). During the present study, although the limited fed individuals did not show any remarkable growth regarding shell length, they actually did have thinner lips during summer and thicker lips during winter. Deposition of shell material on the shell edge of starved whelks was still in progress, but due to a low feeding regime they did not have enough energy to produce an obvious shell increment.

Nassarius reticulatus of the <15 and 15-20 mm size classes that were reared under laboratory conditions with a regular supply of food, had a significantly higher growth rate than marked and recaptured individuals of similar size that were released into the intertidal lagoon. However, larger size classes 20-25 mm and >25 mm maintained under laboratory conditions had similar growth rates to the marked and recaptured gastropods of similar size. Younger and smaller whelks that invest a large amount of energy into somatic and shell growth grew faster in laboratory conditions where the available food supply was presumably higher. In sexually mature individuals, where most of the absorbed energy is invested into reproductive activity and shell increase is reduced, the growth rate in conditions of excess food supply (laboratory conditions) was similar to the growth rate under restricted food supply (field conditions). Spight (1981) reported that *Thais emarginata*, *T. lamellosa* and *T. canaliculata* grew faster under laboratory conditions than in the field. Ota & Tokeshi (2000) also indicated that individuals of *Japeuthria ferrea* and *J. cingulata* rarely achieved their potential maximum growth in the field because of insufficient food, competition and environmental constraints.

This chapter describes the population dynamics of *Nassarius reticulatus* in an intertidal lagoon and a subtidal beach in Rhosneigr (Anglesey). The catchability of *N. reticulatus* was highest during the summer months, when seawater temperature was also at a maximum. The intertidal lagoon habitat supported a significantly greater population than the subtidal beach. A similar recruitment pattern was observed in both the intertidal lagoon and subtidal beach. The larvae of *N. reticulatus* emerge between spring and summer and settle from late summer to early autumn. Juveniles of about 7-8.9 mm shell length appeared for the first time in the population during the following February to April. Mortality of *N. reticulatus* was higher during the summer months, and the percentage of annual survival rate was lower for the intertidal lagoon population. The growth pattern described from the MPA analysis is closer to the growth increments of the marked and recaptured gastropods that were released into the intertidal lagoon, than the LFDA analysis results. The MPA analysis of the length-frequency distributions indicated that the growth pattern of *N. reticulatus* was different in the intertidal lagoon and on the subtidal beach. Gastropods from the intertidal lagoon reached a higher asymptotic maximum length (L_∞) and had a lower growth constant (K) than animals from the subtidal beach. A strong seasonality in growth of *N. reticulatus* was observed in both habitats. However, growth of the intertidal lagoon whelks decreased after the end of September and resumed at the beginning of April, about one month later than growth of the subtidal beach gastropods. Even when *N. reticulatus* were maintained under the same controlled laboratory conditions for a period of 6-7 months, a significant difference was found between the growth pattern of whelks that were originally collected from the intertidal lagoon or the subtidal beach. Growth of animals maintained under laboratory conditions was temperature and size dependent. Growth was higher during the warmer months and smaller size classes grew faster than older whelks. Growth rates were similar between female and male *N. reticulatus*, although females attained a larger shell length. Low food availability did not affect survival of *N. reticulatus* in the laboratory, but growth of whelks was significantly suppressed. Regularly fed animals in the laboratory grew more than individuals from the natural environment.

CHAPTER 4

The use of statoliths for age determination of *Nassarius reticulatus*

4.1 INTRODUCTION

Early attempts to determine the age of gastropods relied mainly on morphological characteristics of the shell, such as shell length, thickness of shell lip and the formation of teeth on the inner lip margins. Populations of *Nucella lapillus* were separated into age-classes based on the length of the shell for the first 3 years of life, while in older individuals, where shell growth was considerably slower, age estimations were based on the thickness of the shell lip and the presence of teeth on the inner lip (Berry & Crothers, 1968; Feare, 1970a; Coombs, 1973). Previous studies have attempted to age adult *Strombus gigas* on the basis of lip thickness (Wood & Olsen, 1983; Appeldoorn, 1988). However, estimating the age of gastropods based on lip thickness can be quite precarious either due to the expected overlap between adjacent age-classes (Coombs, 1973) or due to increased erosion of the edge of the lip over time (Appeldoorn, 1988; Stoner & Sandt, 1992). Tallmark (1980) attempted to estimate the growth and age of *Nassarius reticulatus* by locating the previous year ring on the shell surface and measuring the thickness of the outer lip. Although the first three year-classes were distinct, after the fourth year growth rates were decreased or growth had completely ceased, thus age determination based on shell rings was not possible (Tallmark, 1980).

The external surface of the shell of several archaeogastropod species has revealed distinctive annual growth marks that have been used for studying growth patterns and for age determination. External annual growth marks have been observed on the top shell *Monodonta lineata* (Williamson & Kendall, 1981). Two growth rings, one dark and one light, are formed annually on the keyhole limpet *Fissurella crassa* shell (Bretos, 1980) and on the patellid limpet *Nacella (Patinigera) cancinna* shell (Picken, 1980). The dark ring is formed during winter as a result of a decrease in temperature, and the light ring is formed during the spawning period due to changes in growth (Picken, 1980). However, for most gastropod species the outer shell surface offers little information based on shell marks or rings that could be used for age estimation (Richardson, 2001). Barroso *et al.* (2005a) attempted to age *Nassarius reticulatus* from rings on the external surface of the shell and noted that they became less discernible as the gastropods increased in size, thus resulting in an underestimation of age. The shell margin is frequently damaged and repaired resulting in the production of growth checks, which can be difficult to distinguish from those of annual origin (Richardson *et al.*, 2005b). Sectioning of the shell in abalone species, such as *Haliotis rubra* (Prince *et al.*, 1988a), *H. midae* (Erasmus *et al.*, 1994), *H. mariae* (Shepherd *et al.*, 1995a), *H. fulgens* (Shepherd *et al.*, 1995b), and in the limpet *Patella vulgata* (Ekaratne & Crisp, 1982; 1984) revealed a series of internal micro-growth bands. In *H. fulgens* four growth lines are deposited during the first year and three lines are deposited in each subsequent year (Shepherd *et al.*, 1995b), whereas in *H. midae* three lines were deposited in the first year and one line in each following year (Erasmus *et al.*, 1994). Growth lines are deposited during periods of maximum or minimum seawater temperatures or during the spawning season (Shepherd *et al.*, 1995a; 1995b). In *P. vulgata* age was estimated from micro-growth bands in the shell which are formed during every tidal emersion (Ekaratne & Crisp, 1984). Suppression of the width of the growth increments in summer was probably affected by gametogenesis (Ekaratne & Crisp, 1984). The conical spired shell of mesogastropods and neogastropods is not suitable for producing a continuous growth sequence without repeated serial sectioning and complicated interpretations of the sections, thus few studies have been presented on these species concerning shell growth lines (Richardson, 2001). Ekaratne & Crisp (1982; 1984) estimated the age of *Littorina littorea* and *Nucella lapillus* by measuring the tidal micro-growth bands in the newly formed lip at the margin of the aperture of the shells. Seasonal changes in the width of the growth bands were mainly influenced by temperature (Ekaratne & Crisp, 1984). In a recent study sections along the axis of maximum growth in shell lip of adult *N. reticulatus* revealed a series of fine micro-growth bands deposited perpendicular to the shell surface and it was assumed that growth cessation in winter caused the formation of an annual internal dark line that was subsequently used for age estimation (Barroso *et al.*, 2005a).

A new development in the study of growth and ageing of gastropods is the identification of growth increments in statoliths. Statoliths are structures similar to fish otoliths, that are formed of aragonitic calcium carbonate deposited on a protein matrix and they exhibit periodic growth increments (Radtke, 1983). Pannella (1971) indicated that concentric rings are deposited in otoliths of cold-temperate fish with a daily periodicity. Radtke *et al.* (1985) reported the presence of distinct annual rings in the otoliths of oyster toadfish *Opsanus tau*, together with daily micro increments. Since then several studies have used otoliths for age determination and have suggested that daily formation of otolith increments could be based on environmental and physiological factors such as photoperiod, temperature, feeding and growth, synchronised with an endogenous cicardian rhythm (Tanaka *et al.*, 1981; Campana & Neilson, 1985).

Analysis of otolith growth increments can give ecological information about events in the life history of fish larvae, such as time spent in the plankton, dispersal distance of larvae and the exact age in days at which a habitat shift from pelagic to demersal phase occurs (Victor, 1982). A mark corresponding to settlement of the planktonic larvae onto the reef was found in the otoliths of the coral-reef wrasse *Thalassoma bifasciatum* (Victor, 1982). Also the first prominent ring observed on the otolith of *Brevoortia tyrannus* larvae was related to hatching and the second prominent ring observed was related to the shift from yolk absorption to exogenous feeding (Maillet & Checkley, 1991). Moksness & Fossum (1991) suggested that analysis of otolith increments could result in the identification of spring- and autumn-spawned North Sea herring, based on differences in the distance between the hatching mark and the nucleus. Spring spawned larvae would have a greater distance between the hatching mark and the nucleus and wider increments on their otoliths than autumn spawned larvae. Maillet & Checkley (1991) found that storm events resulted in decreased widths of otolith increments in larvae of the Atlantic menhaden *B. tyrannus* due to dispersion of food. Furthermore, otolith increment micro-structure has been used to estimate growth variation due to temperature (Gutierrez & Morales-Nin, 1986), to identify different stocks (Campana & Casselman, 1993), to reveal past water temperatures from isotope analysis (Devereux, 1967), or to determine survivorship curves and mortality estimates (Crecco *et al.*, 1983). Relationships between somatic growth and otolith growth have been used to back-calculate growth rates from otolith increments and to construct an age-length curve (Methot, 1981; Wright *et al.*, 1990). Devereux (1967) proposed that temperature archives derived from oxygen isotopic analysis of fish otoliths could reveal information about the fish habitat, such as depth were the fish was living and possible migration patterns of the population in other regions.

Statoliths have been extensively used, especially during the last 20 years, as tools for ageing several cephalopod species. Young (1960) was the first to observe periodic increments in the statoliths of the myopsid squid *Alloteuthis subulata*, and later Clarke (1966) also noted the presence of increments in the statolith of ommastrephid squids *Ommastrephes caroli*, *O. pteropus*, and *Todarodes sagittatus*. However, Lipinski (1978) and Spratt (1978) were the first to analyse the growth increments in statoliths and use them to estimate the age of *Illex illecebrosus* and *Loligo opalencens* respectively. Since then growth lines and patterns of growth increments have been extensively studied in the statoliths of several species of squids and sepioids (Jackson, 1994). For squid and sepioid species deposition of growth increments is daily and this has been demonstrated in many species such as *Gonatus fabricii* (Kristensen, 1980), *I. illecebrosus* (Radtke, 1983; Dawe *et al.*, 1985), *Alloteuthis subulata* (Lipinski, 1986), *Sepioteuthis lessoniana* (Jackson, 1990a), *Idiosepius pygmaeus* (Jackson, 1989; Jackson & Choat, 1992), *Loliolus noctiluca* (Jackson, 1990b; Jackson & Choat, 1992; Dimmlich & Hoedt, 1998), *Loligo chinensis* (Jackson, 1990b) and *Loligo vulgaris* (Lipinski *et al.*, 1998). Although the growth increments near the nucleus in statoliths of *Sepia hierreda* are deposited daily, the formation of thicker and more widely spaced increments (on the outer zones of the lateral dome of the statolith), is probably based on a lunar or seasonal rhythm (Raya *et al.*, 1994). Kristensen (1980) suggested that growth increments in the statoliths of *G. fabricii* in the first zone after deposition of the nucleus are deposited daily, whereas the increments in the second and third zones are deposited fortnightly and monthly, respectively. Similarly, deposition of statolith increments was not daily for the embryo of *A. subulata* and it was suggested that increment formation is controlled by temperature and light cycles (Morris, 1991). The mechanism of increment formation in statoliths is not yet understood, although there is clear evidence of a daily deposition in many cephalopod species (Rodhouse & Hatfield, 1990). Increments in squid statoliths are produced under laboratory conditions in the absence of tidal, feeding, temperature or migration cycles (Dawe *et al.*, 1985), suggesting that their formation is dependent on an endogenous circadian rhythm (Rodhouse & Hatfield, 1990).

Lipinski *et al.* (1991) suggested that a "growth ring" consists of one light and one dark ring, and these two together should be referred to as a "growth increment". Bettencourt & Guerra (2000) indicated that the dark ring of squid statoliths is rich in organic matter and is deposited during the day, and the light ring is rich in $CaCO_3$ and is deposited during night. Morris (1991) suggested that the low concentration of Mg^{2+} ions in the squid statocyst fluid allows the $CaCO_3$ precipitation in the form of aragonite and this process is controlled by the pH of the statocyst endolymph and the organic matrix. However, according to Lipinski (1993) periodic changes in strontium concentration in the statocyst fluid (possibly related to squid activity levels) can either promote or inhibit calcification, thus leading to the formation of growth increments.

Arkhipkin & Bizikov (1997) proposed that since increments are deposited daily in most squid species statoliths, they can give information regarding their age (number of increments), growth rate (width of increments), ontogenetic events (interruptions-checks) and shifts (change of habitat zone) during the life cycle of the animal. Kristensen (1980) described 3 zones in the statoliths of *Gonatus fabricii*, a post-nuclear light zone, followed by a dark zone and a third outer zone with prominent increments, and he suggested that these zones might represent different maturity stages. Morris & Aldrich (1985) noted three zones on the statoliths of *Illex illecebrosus*. The first one was translucent and thought to represent pre-hatching growth (although later Balch *et al.* (1988) showed that the first zone was formed after hatching), the second zone was opaque and thought to represent growth until maturity and the third was translucent and thought to represent mature growth. Radtke (1983) suggested that the stable isotopic composition of the statoliths of the squid *I. illecebrosus* may contain information about the environmental past of individuals. Oxygen isotopes are deposited in isotopic equilibrium with the surrounding environment and are dependent on temperature and oxygen in the seawater, while carbon deposition seems to be related to biological processes (Radtke, 1983).

Proper statolith preparation, increment identification and subsequent reading are crucial for obtaining accurate age estimates (Lipinski *et al.*, 1998). Useful reviews for statolith preparation, increment counting by light microscopy or scanning electron microscopy have been provided by Jackson (1994). Statoliths of small species can be mounted whole in a histological mounting medium such as DPX (dibutyl-phthalate-polystyrene-xylene) or even in a thermoplastic cement such as Crystal Bond and the increments can be directly observed under a microscope (Jackson, 1989; 1990a). However, statoliths of larger species often require grinding and polishing in order to observe the increments (Jackson, 1990a; 1990b; Lipinski *et al.*, 1998), although it has been noted that after sectioning growth increments in statoliths fade on prolonged exposure to air (Rodhouse & Hatfield, 1990). Confirmation of the daily periodicity of statolith increment formation has been achieved by placing a chemical mark in the statolith and then comparing the number of days elapsed with the number of increments produced after marking (Rodhouse & Hatfield, 1990; Jackson, 1994). Increments on cephalopod statoliths can be marked by injection or immersion in tetracycline hydrochloride (Dawe *et al.*, 1985;

Lipinski, 1986; Jackson, 1989; 1990a; 1990b; Lipinski *et al.*, 1998; Dimmlich & Hoedt, 1998), calcein (Jackson, 1990a) or strontium (Dawe *et al.*, 1985; Hurley *et al.*, 1985).

Analysis of growth increments in statoliths is a more direct and a more suitable method for ageing cephalopods than the separation of modal size classes in size-frequency distributions of a population, especially in organisms that migrate (Lipinski, 1980). Migration of animals, daily or seasonally, can cause the presence of mixed age groups in the population, thus resulting in an overestimation of age from the size-frequency analysis (Dawe *et al.*, 1985; Rodhouse & Hatfield, 1990). When Jackson *et al.* (1997) compared age and growth estimations determined by either length frequency data or by statolith increments in *Lolliguneula brevis*, they indicated that length frequency analysis overestimated the life span by a factor of 3 to 7 times. Analysis of size-at-age data derived from statolith increments is a better method for estimating growth and longevity in short-lived and rapid-growing species like cephalopods than length frequency analysis (Jackson & Choat, 1992).

Until recently only a few studies had described the use of growth rings in statoliths as a tool for determining the age of gastropod larvae (Bell, 1982; 1983; 1984; Grana-Raffucci & Appeldoorn, 1997; Zacherl *et al.*, 2003) and adults (Karageorgopoulos, 1997; Saurel, 2002; Barroso *et al.*, 2005b; Richardson *et al.*, 2005a; 2005b). Gastropod statoliths are found in the statocysts, the sense organs for equilibration, which provide the animal with information on the direction of gravity with reference to the animal's position (Dorsett, 1986). In gastropods the statocysts are located between the cerebropleural and pedal ganglia, just anterior to the cerebropedal connectives (Dorsett, 1986). Each statocyst is a spherical cavity with a ciliated inner surface and an outer wall formed from connective tissue (Fretter & Graham, 1994). The statocysts contain statolymph, in which calcareous particles are floating, either one statolith or several smaller statoconia (Fretter & Graham, 1994). Statoconia occur in the primitive archaeogastropods and in some freshwater mesogastropods families, whereas the rest of the mesogastropod families and the neogastropods have a single statolith in each statocyst (Fretter & Graham, 1994).

Estimation of the age of gastropod larvae by means of statoliths could represent a useful tool for understanding aspects of age, growth, life histories and ecology (Grana-Raffucci & Appeldoorn, 1997). Statoliths could be used with some confidence for the assessment of growth rates of field populations, as long as deposition of the rings is periodical and not in response to discrete feeding episodes (Bell, 1983). Bell (1983; 1984) demonstrated that the number of statolith increments corresponded one-to-one with the age in days after hatching in *Littorina scabra* larvae. The number of increments and the diameter of statoliths increased as the larvae grew, while the deposition of increments on the statoliths was not dependent on feeding rates or starvation (Bell, 1983; 1984; Grana-Raffucci & Appeldoorn, 1997). The rate of increment deposition in statoliths of veligers of *Strombus costatus* and *S. gigas* remains constant after hatching and is independent of starvation (Grana-Raffucci & Appeldoorn, 1997). Deposition of statolith material was found to be influenced by the light-dark cycle (Bell, 1983; 1984). When the larvae of *L. scabra* were reared in the laboratory under constant light or constant dark conditions, no growth increments were apparent in their statoliths. By contrast growth increments were formed in statoliths when the larvae were reared under 12h-light and 12h-dark conditions (Bell, 1983; 1984).

Statocysts appeared in four-day old embryos of *Strombus gigas* and were fully functional by day 6 (D' Asaro, 1965). Growth increments in the statoliths of *S. gigas* larvae have been observed by D' Asaro (1965) and Grana-Raffucci & Appeldoorn (1997). Grana-Raffucci & Appeldoorn (1997) described three regions in the statoliths of the queen conch *S. costatus* and *S. gigas* larvae. The three regions were distinguishable by changes in density of the increments, and they could represent differences in metabolism and mineral deposition during the different stages of larval development. The first region, defined as the pre-hatching region, was lighter and wider and consisted of a primordial granule in the center that was surrounded by four increments. The second region was composed of increments formed from hatching to metamorphosis and was darker with thinner increments than the first region. The third region appeared after metamorphosis in juveniles of *S. gigas* only and was lighter with wider increments than the second region. The mean number of increments formed one day before metamorphosis (20 days old) was 26.06, and two days post-metamorphosis (23 days old) 29.40 increments were counted (Grana-Raffucci & Appeldoorn, 1997). The growth of statoliths is influenced by environmental or genetic factors and as a result the relationship between shell length and statolith diameter is not linear for the entire larval and juvenile life (Grana-Raffucci & Appeldoorn, 1997). The utilisation of daily increments in larval statoliths can contribute to studies and compare growth rates between wild and laboratory-reared populations, larval nutrition, duration of larval life in the plankton, dispersal patterns and competency to metamorphose (Bell, 1982). Recently statoliths of *Concholepas concholepas* larvae have been used as natural tags of natal origin for discrimination between larvae from geographically separated sites (Zacherl *et al.*, 2003). The trace elemental composition (Ba/Ca, Zn/Ca and Pb/Ca) of the natal core in larval statoliths appeared to be site-specific, and that could be due to a number of causes such as variations in elemental concentrations in seawater, seawater temperatures, genetics, salinity and variable precipitation rates that have yet to be studied (Zacherl *et al.*, 2003).

Statoliths of adult gastropods have only recently been examined in *Buccinum undatum* (Karageorgopoulos, 1997), *Nucella lapillus* (Saurel, 2002), *Nassarius reticulatus* (Barroso *et al.*, 2005b), *Polinices pulchellus* (Richardson *et al.*, 2005a) and *Neptunea antiqua* (Richardson *et al.*, 2005b). Statoliths in *B. undatum* and *N. lapillus* have a smooth surface and are ellipsoid in shape with numerous dark and light concentric rings (Karageorgopoulos, 1997; Saurel, 2002). The dark rings in *B. undatum* statoliths were narrow and represented zones of discontinuous growth, while the light rings were wider and represented zones of incremental growth (Karageorgopoulos, 1997). Three regions were observed in the statoliths of *N. lapillus* which differ in terms of increment density and composition in organic material and calcium carbonate (Saurel, 2002). The first region was formed before hatching of juveniles and was rich in organic material, the second region was probably formed during the first year of life and was also rich in organic material but had more compressed increments, while the third region contained more calcium carbonate and was formed in statoliths of older mature animals (Saurel, 2002).

Attempts have been made to explain ring formation in the statoliths during these early studies but no strong experimental evidence has yet been provided. The first ring formed on the statolith was related either to the hatching of the juvenile from the egg capsules as observed in species with no planktonic stage (direct development) (*Buccinum undatum*, Karageorgopoulos, 1997; *Nucella lapillus*, Saurel, 2002), or to the settlement and metamorphosis of larvae in species with a preliminary planktonic phase (planktotrophic development) (*Polinices pulchellus*, Richardson *et al.*, 2005a; *Nassarius reticulatus* Barroso *et al.*, 2005b). However, Zacherl *et al.* (2003) has suggested that the first prominent ring on the larval statolith of *Concholepas concholepas* was formed during hatching, and not during settlement. Larvae of *C. concholepas* also spend a period of 3 months in the plankton as veligers before settlement and metamorphosis (planktotrophic development) (Zacherl *et al.*, 2003). Subsequent prominent rings in the statoliths of *B. undatum* and *N. lapillus* were thought to be of annual origin, although there was no experimental evidence to support these interpretations (Karageorgopoulos, 1997; Saurel, 2002). Based on comparisons of the estimated shell length at formation of statolith rings with length-at-age data derived from length frequency analysis Richardson *et al.* (2005a) and Barroso *et al.* (2005b) suggested that the statolith rings in *P. pulchellus* and *N. reticulatus* respectively were formed annually.

The present study is a detailed experimental approach in order to determine the periodicity of the rings in the statoliths of *Nassarius reticulatus* and to use the statolith growth increments to reveal information about life-history, growth and recruitment patterns of the species. The aims of this chapter were: 1) to investigate the timing of formation and the variability in diameter of the first statolith ring, 2) to determine the periodicity of the prominent rings and to validate their use as a tool for estimating the age of *N. reticulatus*, 3) to determine the time of year that the growth rings are formed, 4) to establish a relationship between statolith diameter and shell length in order to estimate shell length at the formation of annual rings (length-at-age data), 5) to examine the variation in shell length and statolith diameter during settlement of *N. reticulatus* from different localities in order to determine differences in recruitment patterns, 6) to investigate possible differences in the growth patterns of males, females and imposex affected animals from different areas, and 7) to validate the use of statolith rings as an appropriate technique for the estimation of past shell length, using marked individuals for the monitoring of growth.

4.2 MATERIALS AND METHODS

4.2.1 Sampling

Nassarius reticulatus were collected subtidally and intertidally using baited traps as described in chapters 2 (section 2.2.1) and 3 (section 3.2.1) respectively. *N. reticulatus* were collected from 6 different areas around Anglesey: 120 animals from an intertidal lagoon in Rhosneigr (8.2-30.8mm), 40 animals from Rhosneigr beach (15.6-27.8mm), 40 animals from Malltraeth beach (12.3-31.4mm), 40 animals from Church Bay (11.4-21.1mm), 39 animals from Carmel Head and Cemlyn Bay (14.6-26.2mm) and 13 animals from Bull Bay (15.4-26.2mm). The location of the sampling sites is shown in chapters 2 and 3 (figures 2.2 and 3.1), and a detailed description of the sites can be found in section 2.2.2. Samples from the Rhosneigr intertidal lagoon were collected during June 2003 (40 animals, size range 8.3-27.9mm), August 2003 (40 animals, size range 9.9-30.8mm) and February 2004 (40 animals, size range 8.2-29.6mm). All samples from the other areas were collected during July 2003. An additional sample of 40 animals covering a wider size range (6.4-30.0mm), and containing smaller individuals, was collected from the Rhosneigr beach in November 2003 and was used to extend the range of juvenile whelks studied.

The samples collected in June, August and February from Rhosneigr lagoon were used to investigate whether there might be seasonal variations in the relationship between shell length and statolith diameter, and to establish during which season the dark rings are deposited in the statoliths. The samples from all 6 areas (including the lagoon) were compared in order to investigate potential regional differences in the statolith growth patterns. An equation between statolith diameter and shell length was established and used to estimate the shell lengths of *Nassarius* during ring formation. The estimation of shell length using the statolith rings was validated using the statoliths of marked animals, for which growth had been monitored for a period of about 2 years. Twenty one of the marked *N. reticulatus* that were released in the intertidal lagoon (details in chapter 3, section 3.2.4) and recaptured between August and September 2004 were sacrificed and their statoliths extracted.

4.2.2 Statolith extraction from adult *Nassarius reticulatus*

Whelks were transferred to the laboratory for examination of their morphological characteristics (chapter 2, section 2.2.6) and identification of their sex and imposex stage (chapter 7, section 7.2.2-7.2.4). After dissection *Nassarius reticulatus* were stored in the freezer until required for statolith extraction. Rodhouse & Hatfield (1990) indicated that statoliths from squid kept in the freezer for 18 months could be read as easily as those removed from freshly dissected specimens. Kristensen (1980) noted that preservation of squid statoliths in formalin should be avoided because it damages the statolith structure. Prior to dissection the animals were thoroughly defrosted at room temperature. After discarding the viscera and the operculum each animal was placed in a small glass petri dish (50 ml) dorsal side up with the foot stretched out and orientated as if in the crawling position. The petri dish was filled with >1M NaOH solution so that the tissues were completely immersed, covered with a lid and placed in a fume cupboard overnight so that the flesh would be sufficiently digested. The statocysts that contain the statoliths are located in the foot tissue and they lie between the cerebropleural and pedal ganglia, just anterior to the cerebropedal connectives (Dorsett, 1986). The cerebral, pleural and pedal ganglia in higher gastropods form a ring that surrounds the gut (Fretter & Graham, 1994) and they can be used as reference points in order to locate the statoliths. The statoliths were isolated under a dissecting microscope (Leica Wild M3Z) by removing the surrounding digested flesh and breaking the statocyst using very fine tweezers (Dumont 5 Inox Biologie). The pair of statoliths was transferred with a Pasteur pipette into a watch glass and rinsed with deionised water. After 2 minutes the statoliths were transferred into another watch glass containing 70% alcohol and allowed to stand for 5 minutes in order to remove any attached tissues. In order to avoid a reduction in the clarity of the statoliths caused by prolonged exposure to air (Dawe *et al.*, 1985), cephalopod statoliths are usually stored in absolute alcohol, 70% ethanol, gelatine capsules or glycerol after extraction until further processing (Rodhouse & Hatfield, 1990). However, due to the small size of the *N. reticulatus* statoliths and their possible loss during handling, the statoliths were processed immediately after extraction.

After rinsing in 70% alcohol the statoliths were placed in a microscopic cavity slide and left to air dry. A plain microscopic slide was warmed above a flame until it reached a temperature >80 °C, and a small amount of CrystalBond^{TM} 509 resin was melted onto the slide. The softening point of the resin is 71°C and the flow point 135°C. Crystal Bond is an excellent mountant as it is completely translucent, and the statoliths can easily be manipulated because it melts quickly at a low temperature and hardens relatively fast after removal from the heat (see Jackson, 1990a). Each of the two statoliths of every pair was transferred with fine tweezers onto a separate slide and immersed in the resin. While the resin was still warm the statolith was orientated with its long axis flat in order to avoid the appearance of double rings (see figure 4.3a). The resin was melted again if repositioning of a statolith was necessary. Due to the clearly identified rings on the statoliths of *Nassarius reticulatus* no grinding or polishing of the statoliths was necessary and the statoliths could be observed immediately under the microscope (Leitz Laborlux S). Each statolith was photographed using a digital camera (ColorView - Soft Imaging System) attached to a microscope. A photo-library was compiled from the digital images of the statoliths and the photos were interpreted with the software package AnalySIS (version 3.2 – Soft Imaging System GmbH 2001). The number of rings was counted and the nucleus diameter, the total diameter of the statolith, and the distance between the rings (growth increments) were measured in all the statoliths.

4.2.3 Statolith extraction from embryos, larvae and juveniles of *Nassarius reticulatus*

Larvae were hatched from egg capsules that had been produced by spawning adult *Nassarius reticulatus* held in the laboratory, and were reared under ambient temperature from hatching until settlement (for details see chapter 6, sections 6.2.1 & 6.2.2). For observations of the larval statoliths sub-samples of 20 larvae were taken at weekly intervals between hatching and settlement. Post-metamorphic juveniles were successfully reared under laboratory conditions up to an age of 500 days (for details see chapter 6, section 6.2.7). Sub-samples of these juveniles (the number depending on availability) were taken at frequent intervals (usually monthly) for observation of the juvenile statoliths. In addition, 20 embryos were isolated from the egg capsules produced from the adult *N. reticulatus* and the size of the embryonic statoliths measured.

The shell length of embryos, larvae and juveniles was measured under a calibrated dissecting microscope (Leica Wild M3Z) from apex to the edge of the siphonal canal. The statoliths were directly visible in the foot tissue under the dissecting microscope (see figure 4.1). The animals were placed in a cavity slide with a few drops of >1M NaOH solution and the shell was crushed by applying gentle pressure. The remains of the shell were carefully removed with fine tweezers and the animals were left in NaOH for a period of 4-5 hours. The digested flesh could then be removed with fine tweezers and the statoliths were easily isolated. The procedure of rinsing the statoliths in deionised water and 70% alcohol, mounting them in resin and further interpretation using image analysis was similar to that described above for the adult statoliths. In some cases due to the very small size of the larval statoliths it was not possible to transfer them into resin, so the diameter of the nucleus, the diameter of the statolith and the growth increments (where they were formed) were directly measured under a calibrated microscope. Representative photographs of the statoliths were taken at various stages of larval development.

4.2.4 Analysis of data

Von Bertalanffy growth parameters were estimated from the length-at-age data using the FISAT II software (version 1.1.3). The comparison of von Bertalanffy growth curves was based on the Hotelling's T^2 statistical test, modified to an F statistic according to Bernard (1981). The asymptotic maximum length (L_∞), the growth constant (K) and theoretical age at which length would have been zero (t_0), as well as the differences in values of the same parameters between the two groups compared are expressed as column vectors,

$$[\vec{P}_1]=\begin{bmatrix} L_{\infty(1)} \\ K_{(1)} \\ t_{0(1)} \end{bmatrix} \quad [\vec{P}_2]=\begin{bmatrix} L_{\infty(2)} \\ K_{(2)} \\ t_{0(2)} \end{bmatrix} \quad [\vec{P}_1-\vec{P}_2]=\begin{bmatrix} L_{\infty(1)}-L_{\infty(2)} \\ K_{(1)}-K_{(2)} \\ t_{0(1)}-t_{0(2)} \end{bmatrix}$$

The T^2 statistic is calculated from the following equation:

$$T^2=\frac{N_1\cdot N_2}{N_1+N_2}\cdot[\vec{P}_1-\vec{P}_2]'\cdot S^{-1}\cdot[\vec{P}_1-\vec{P}_2]$$

where N_1 and N_2 is the number of individuals in the groups compared, $[\vec{P}_1-\vec{P}_2]$ is the column vector of differences between estimates of the same parameters from the two groups, $[\vec{P}_1-\vec{P}_2]'$ is the transpose (row vector) of $[\vec{P}_1-\vec{P}_2]$, and S^{-1} is the inverse of the pooled variance-covariance matrix S, which is estimated from the formula:

$$S=\frac{(N_1-1)S_1+(N_2-1)S_2}{N_1+N_2-2}$$

where S_1 and S_2 are the estimated variance-covariance matrices of L_∞, K and t_0 from the two groups compared. The critical value of T_0^2, which is a modified tabulated F statistic, is used to test the estimated T^2 statistic for significance:

$$T_0^2 = \frac{3 \cdot (N_1 + N_2 - 2)}{N_1 + N_2 - 4} \cdot F \qquad \text{where } a=0.001,\ DF= N_1+N_2-4 \text{ for tabulated F}$$

When T^2 is higher than T_0^2 growth of shell between a pair of areas is described as dissimilar, and when T^2 is lower than T_0^2 growth of shell between a pair of areas is described as similar. If the Hotelling's T^2 test was found significant ($T^2>T_0^2$) the Roy-Bose simultaneous confidence intervals (C.I.) around differences between the parameters were estimated in order to indicate which of the three parameters contributed most to the statistical significance (Morrison, 1976).

$$C.I. = (p_1 - p_2) \pm \sqrt{\frac{N_1 + N_2}{N_1 N_2} \cdot T_0^2}$$

where p_1 and p_2 are the L_∞, K, or t_0 for the first and second group compared. When the confidence intervals do not include zero the corresponding parameter has a significant influence on the differences in growth between the two areas compared.

4.3 RESULTS

4.3.1 Morphology of the statoliths

The pair of statoliths and their position in the foot tissue were visible through the thin larval shells in newly hatched larvae and for up to 5 weeks after hatching (figure 4.1 a-d). Due to the thickening and differentiation of the foot tissues as the juveniles grew older, dissection of the animal and digestion of the flesh with NaOH was required in order to isolate and observe the statoliths (figure 4.1 e-f). The pair of statocysts, each containing a statolith, in adult *Nassarius reticulatus* is located between the cerebropleural and pedal ganglia, just anterior to the cerebropedal connectives. The statoliths were semi-transparent and 3-dimensional with an elliptical shape. Observations of statoliths mounted in resin under the light microscope revealed a pattern of concentric rings around the nucleus of the statolith. The first ring after the nucleus is formed during larval settlement (see section 4.3.4) and it has been termed as the "settlement ring" in the present study. The pattern of rings was very clear in the statoliths of most of the adult *N. reticulatus* examined and no background

Figure 4.1 The statoliths of the larvae of *Nassarius reticulatus* are clearly visible through the shell: a and b) 4-day old larvae, c) 19-day old larvae, d) 31-day old larvae, e and f) due to differentation of the foot tissues digestion of the flesh with NaOH is needed in order to observe the statoliths of juveniles after settlement. e: eyes, st: statolith, f: foot, v:velum, r:radula

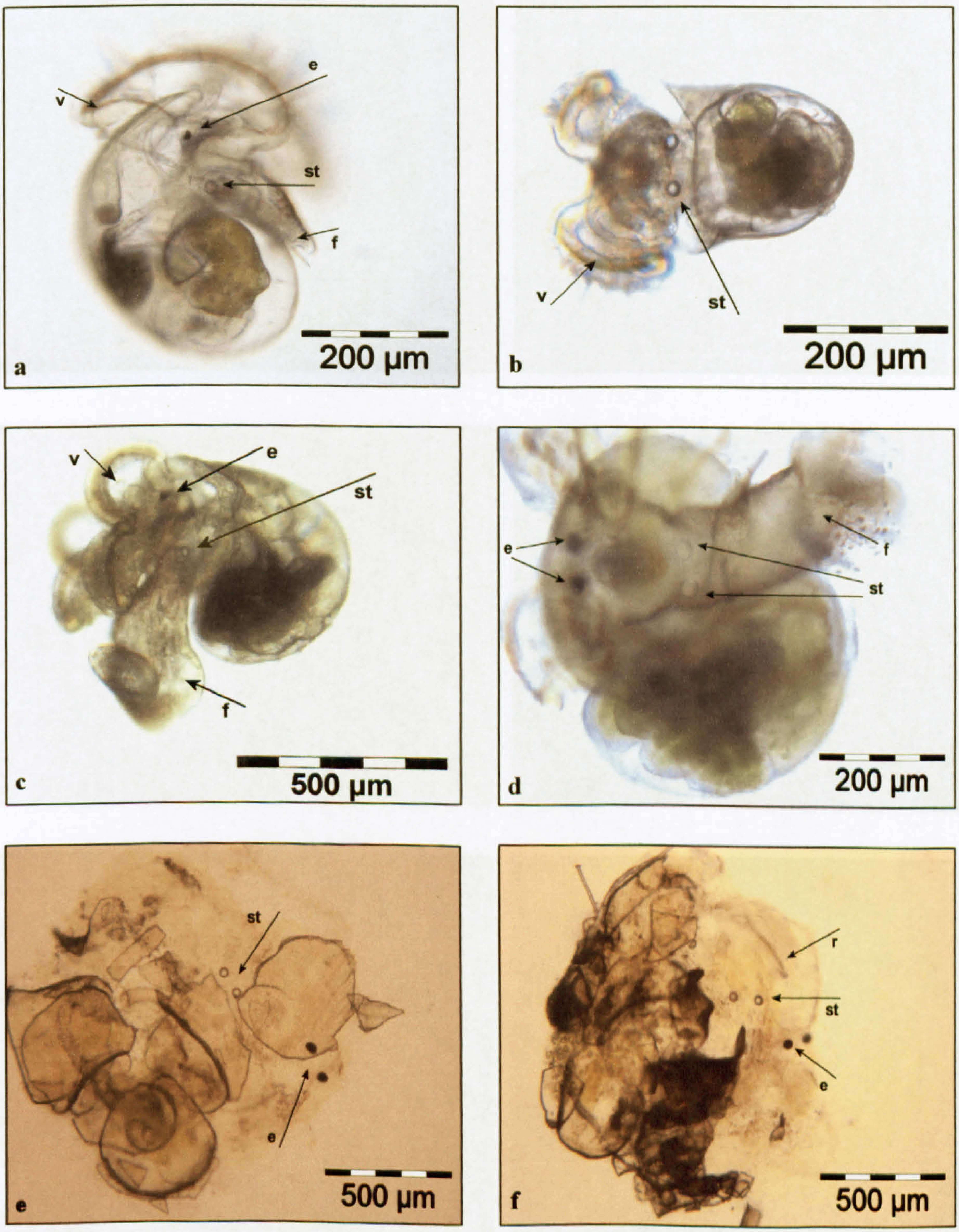
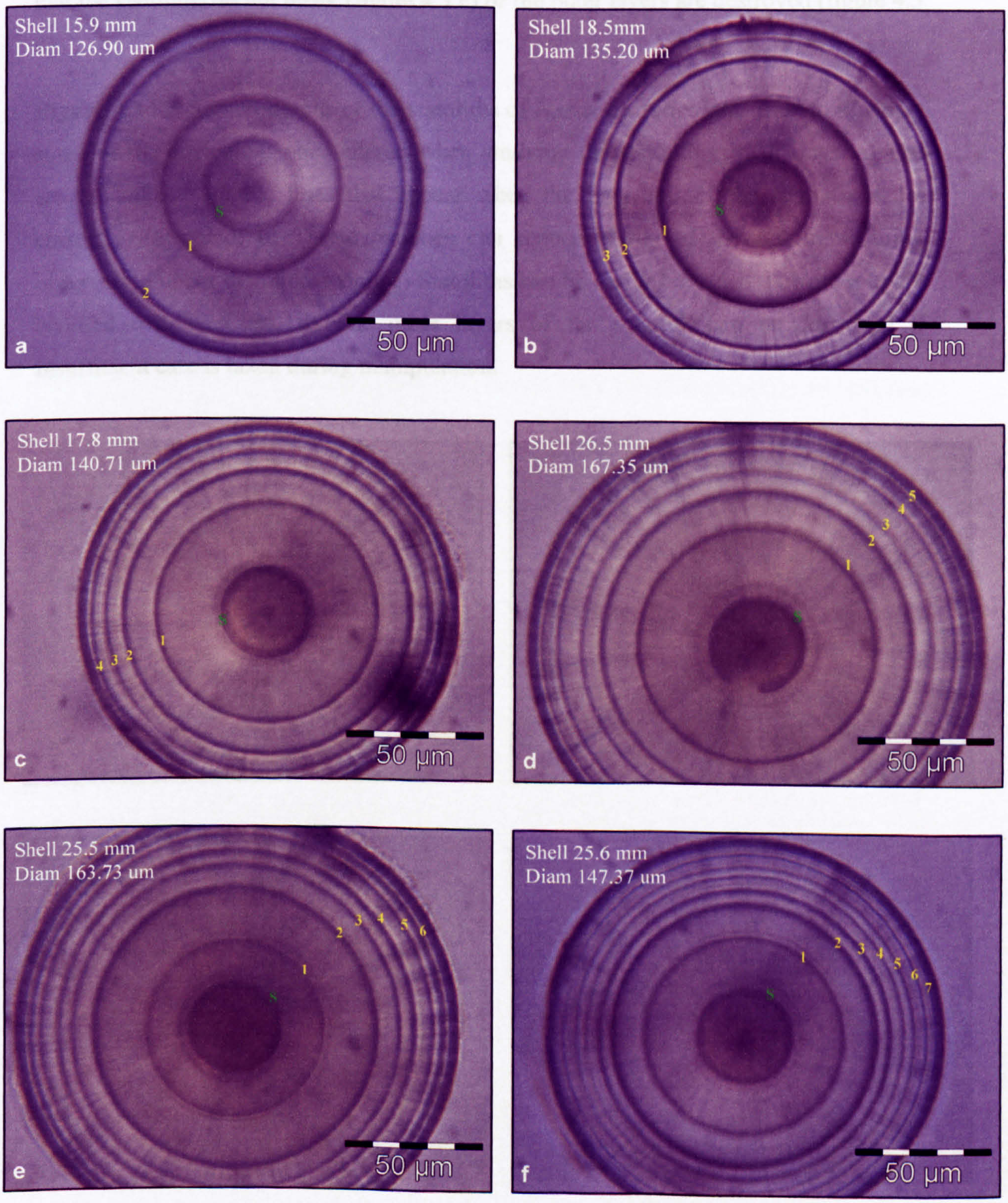
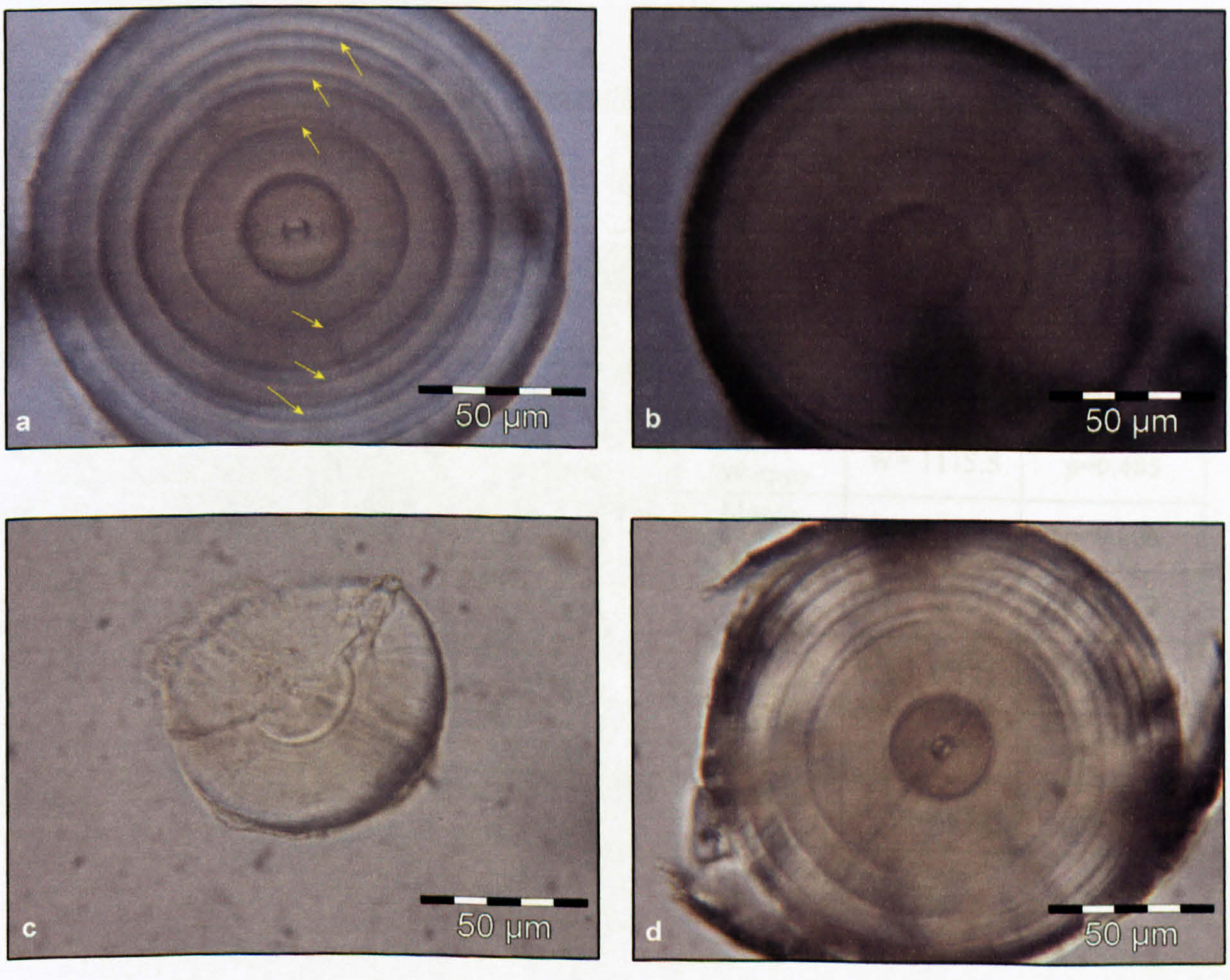


Figure 4.2 Photomicrographs of the statoliths of adult *Nassarius reticulatus*. The settlement ring (S) and the annual rings (numbers 1 to 7) on the statoliths: a) two annual rings, b) three annual rings, c) four annual rings, d) five annual rings, e) six annual rings, f) seven annual rings. The shell length (mm) and statolith diameter (um) are noted for each statolith.



lines were observed. Statoliths containing up to 7 rings were found in some individuals (figure 4.2 a-f). Appropriate orientation of the statolith in the resin with its long axis flat was important in order to avoid the appearance of elliptical and double rings (figure 4.3 a). Also thorough cleaning of the statoliths before mounting them into resin was essential in order to remove adhering tissue residues that could reduce the clarity of the ring patterns (figure 4.3 b). Manipulation of the statoliths has to be done carefully so that the statoliths do not break (figure 4.3 c) or the outer layers are destroyed (figure 4.3 d).

Figure 4.3 Photomicrographs of the statoliths of *Nassarius reticulatus* used to illustrate some of the difficulties encountered when studying the statolith growth rings. a) The arrows indicate double lines that appear when the long axis of the statolith is not correctly orientated, b) Tissue residues can reduce clarity of the statoliths during observation under the microscope, c) Statoliths can be easily broken if they are roughly handled with tweezers, d) The outer layers of the statolith can be destroyed if insufficient care is taken during manipulation.



4.3.2 Comparison of the pair of statoliths

Nassarius reticulatus has a pair of statoliths, each located in a separate statocyst, that lies between the cerebropleural and pedal ganglia within the foot tissue. In some samples (~10%) only one of the two statoliths could be used because the other was either destroyed during manipulation or lost during extraction and mounting. Sometimes (in ~20% of statoliths) the ring patterns were clearer in one statolith than in the other. On these occasions the statolith with the clearest rings was selected for measurements. In order to investigate if there were any significant differences between the two statoliths and to ensure that either of the two statoliths could be used in the analysis, the statolith diameters and the rings diameters of 34 pairs of statoliths from a range of different sized whelks were measured and compared. When the data were normally distributed and had equal variances a two-sample t-test was performed, whereas for non-normal data and equal variances a Mann-Whitney test was performed (table 4.1). No significant differences were found in the diameter of the statolith and the diameter of all the rings between the two statoliths of each pair, thus demonstrating either of the two statoliths could be used for measurements.

Table 4.1 Summary of comparisons between the total diameters and the ring diameters of 34 pairs of statoliths.

	Normality	**Equal variances**	**Test used**	**Test value**	**Probability**
Total diameter	A^2= 0.529, p=0.164 A^2= 0.706, p=0.059	Bartlett's =0.933 p=0.842	2 sample t-test	T= -0.01	p=0.993
Settlement ring	A^2= 0.176, p=0.916 A^2= 0.310, p=0.537	Bartlett's =1.098 p=0.790	2 sample t-test	T= -0.58	p=0.561
1st ring	A^2= 0.508, p=0.186 A^2= 0.828, p=0.029	Levene's =0.354 p=0.554	Mann-Whitney	W= 1115.5	p=0.485
2nd ring	A^2= 0.883, p=0.021 A^2= 0.854, p=0.025	Levene's =0.062 p=0.804	Mann-Whitney	W= 1130.5	p=0.606
3rd ring	A^2= 0.536, p=0.158 A^2= 0.482, p=0.216	Bartlett's =1.056 p=0.876	2 sample t-test	T= -0.32	p=0.752
4th ring	A^2= 0.286, p=0.596 A^2= 0.393, p=0.351	Bartlett's =1.062 p=0.884	2 sample t-test	T= -0.14	p=0.892
5th ring	A^2= 0.254, p=0.688 A^2= 0.242, p=0.730	Bartlett's =1.077 p=0.884	2 sample t-test	T= -0.06	p=0.951
6th ring	A^2= 0.290, p=0.537 A^2= 0.163, p=0.918	Bartlett's =1.014 p=0.984	2 sample t-test	T= 0.08	p=0.939

4.3.3 Statolith rings in adult *Nassarius reticulatus*

The mean diameters of the settlement and annual rings of *Nassarius reticulatus* collected from 6 different locations around Anglesey are presented in table 4.2. Comparisons of the settlement and annual ring diameters between whelks from different areas were performed using a one-way ANOVA test when the data were normal and had equal variances, a Kruskal Wallis test when the data were not normal but had equal variances and a Mood's Median test when the data were normal but did not have equal variances. Comparisons were made only up to the fifth ring because only the animals collected from the Rhosneigr lagoon had formed more rings and only one individual from Carmel Head-Cemlyn Bay had 6 rings.

Table 4.2 Mean diameters (um) (±S.E.) of the settlement ring and annual rings in statoliths of *Nassarius reticulatus* from 6 locations around Anglesey. The mean diameter of each statolith ring in the whelks from all locations was estimated. n.a.: not applicable, only one animal with the respective number of rings found. * indicates significant differences between rings

Location	**Settlement ring**	**ring 1**	**ring 2**	**ring 3**	**ring 4**	**ring 5**	**ring 6**	**ring 7**
Rhosneigr lagoon	33.24 (±0.21)	82.51 (±0.70)	109.53 (±0.68)	124.27 (±0.89)	137.04 (±1.16)	147.09 (±1.54)	152.3 (±2.47)	159.48 (±4.84)
Malltraeth	33.01 (±0.47)	75.83 (±1.31)	116.71 (±1.27)	137.85 (±1.78)	145.88 (±1.82)	152.81 (±4.16)		
Rhosneigr beach	33.26 (±0.33)	87.24 (±1.59)	118.57 (±1.28)	135.37 (±1.47)	146.06 (±1.60)	153.25 (±3.10)		
Bull Bay	33.00 (±0.30)	83.55 (±3.57)	121.34 (±2.82)	141.21 (±2.54)	148.32 (±4.46)			
Church Bay	33.09 (±0.33)	84.81 (±1.57)	118.88 (±1.22)	132.76 (±1.34)	139.18 (±1.49)			
Carmel/ Cemlyn	33.32 (±0.33)	83.35 (±1.31)	120.57 (±2.03)	142.31 (±1.77)	150.13 (±1.84)	147.87 (n.a.)	155.53 (n.a.)	
Mean (all areas)	33.19 (±0.13)	82.68 * (±0.55)	114.89* (±0.56)	131.79* (±0.72)	141.86* (±0.77)	148.11 (±1.34)	152.41 (±2.39)	159.48 (n.a.)

No significant difference was observed in the diameter of the settlement ring (33.19 um) between the different locations (Kruskal Wallis H=0.60, p=0.988). However, comparisons of the diameter of the annual rings 1 to 4 indicated significant differences between whelks from different areas (ring 1: Mood's Median x^2=23.83, p<0.001; ring 2: Kruskal Wallis H=74.65, p<0.001; ring 3: Kruskal Wallis H=95.76, p<0.001; ring 4: Kruskal Wallis H=41.98, p<0.001). The mean statolith diameter during the formation of the first ring ranged from 75.83 um in whelks from Malltraeth to 87.24 um in whelks from Rhosneigr beach, at the second ring from 109.53 um in whelks from Rhosneigr lagoon to 121.34 um in whelks from Bull Bay, at the third ring from 124.27 um in whelks from Rhosneigr lagoon to 142.31 um in whelks from Carmel Head-Cemlyn Bay area, and at the fourth ring from 137.04 um in animals from Rhosneigr lagoon to 150.13 um in whelks from Carmel Head-Cemlyn Bay. *Nassarius reticulatus* with 5 rings were only found in the Rhosneigr lagoon and beach, in Malltraeth and in Carmel Head-Cemlyn Bay area, and no significant differences (Anova F=1.15, p=0.340) were found in the diameter of this ring (148.11 um) between the 4 areas.

4.3.4 Formation of the nucleus, settlement ring and first ring in the statolith

The statoliths of larvae and juveniles of known age hatched in the laboratory were used to investigate at what stage in the whelk's life the rings were deposited. Measurements of the statolith diameter and the shell length at different developmental stages in *Nassarius reticulatus* larvae and juveniles are shown in table 4.3 and summarised in figure 4.5. For comparison, the mean diameters of the settlement and first rings in the adult statolith were determined from animals collected from all the sampling sites (see table 4.2). The mean shell lengths of adults during the formation of the settlement and first rings were estimated from the statolith increments (see section 4.3.9.2, table 4.7).

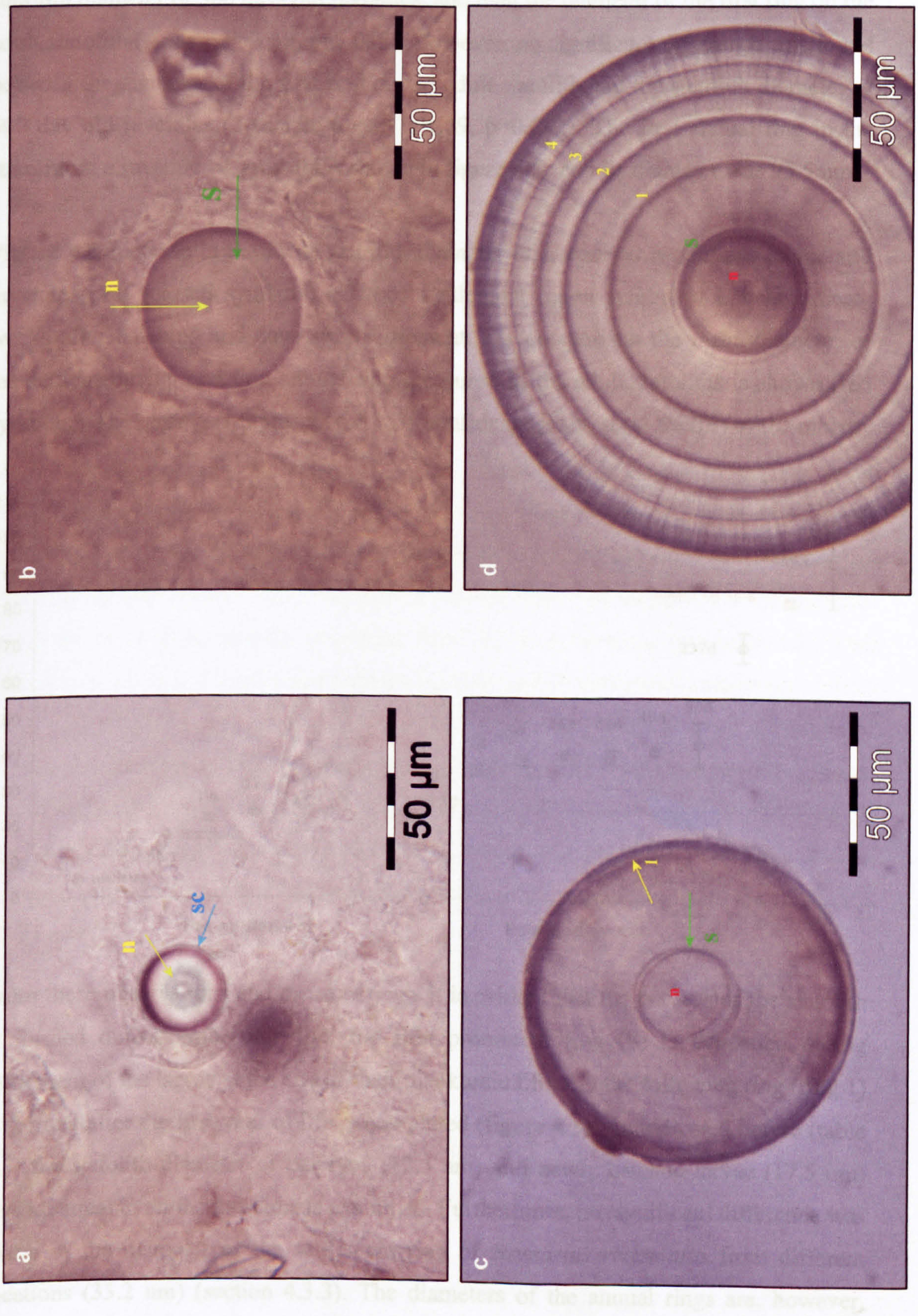
The mean diameter of the statolith nucleus was estimated in both larvae and juveniles of different age and was measured at 5 um in all individuals examined. The mean statolith diameter of the *Nassarius reticulatus* embryos was measured at 12.5 um, and thus it can be inferred that the statolith nucleus is formed during the early embryonic stages. The mean statolith diameter for newly hatched larvae was 17.5 um and for 1-day settled larvae it was 34.0 um. From measurements of the mean diameter of the first prominent ring (S) in the adult statolith (33.2 ±0.13 um) (see table 4.2) and measurements of the first ring in the 1-day settled larvae (34 ±1.15 um) it is clear that the first ring is formed at the settlement of the larvae, rather than during the hatching phase. No significant difference was found between the statolith diameter of the larvae at settlement and the diameter of the settlement ring (S) in adults (2-sample t-test: T=0.73, p=0.467). No rings were observed in the statoliths of larvae before settlement (figure 4.4a), whereas in statoliths of larvae that had settled the first prominent ring could be clearly seen (see figures 4.4 b and c). In statoliths of juveniles older than 132 days (94 days from settlement) a clear settlement ring was observed. The mean diameter of the settlement ring in juveniles was 31.4 um (±0.9) and was not significantly different (2-sample t-test: T=1.38, p=0.168) from the mean diameter of the settlement ring (33.2 ±0.13 um) estimated from the adult statolith (see table 4.2).

Table 4.3 Measurements of mean statolith diameter (±S.E.) (um) and mean shell length (±S.E.) (mm) in larvae and juvenile *Nassarius reticulatus* of known age. Nucleus diameter was estimated from both larvae and juveniles of different ages. Mean statolith diameter and shell length of adults during the formation of the settlement and first ring are also presented. n.a. not applicable, the diameter of nucleus, embryonic statolith and larval statolith upon hatching were identical in all individuals examined.

Stage	Total age (days)	Diameter (±S.E.) (um)	Shell (±S.E.) (mm)
Nucleus	-	5.0 (n.a.)	-
Embryos	-	12.5 (n.a.)	0.25 (±0.005)
Hatching	1	17.5 (n.a.)	0.33 (±0.003)
1 week	7	22.2 (±0.46)	0.40 (±0.005)
2 weeks	14	24.0 (±0.29)	0.50 (±0.006)
3 weeks	21	26.6 (±0.44)	0.61 (±0.009)
4 weeks	28	28.6 (±0.33)	0.67 (±0.010)
Adult settlement ring	(unknown)	33.2 (±0.13)	0.83 (±0.007)
Settlement 1 day	55	34.0 (±1.15)	0.78 (±0.012)
Settlement 2 days	23	36.0 (±0.50)	1.05 (±0.019)
Settlement 9 days	30	38.6 (±0.60)	1.07 (±0.02)
Settlement 15 days	36	40.6 (±0.53)	1.15 (±0.03)
Settlement 31 days	64	40.0 (±1.03)	1.16 (±0.05)
Settlement 49 days	70	41.6 (±0.64)	1.21 (±0.03)
Settlement 94 days	132	43.5 (±3.28)	1.41 (±0.20)
Settlement 237 days	270	70.0 (±2.00)	2.97 (±0.32)
Adult 1st ring	(unknown)	82.7 (±0.55)	5.98 (±0.09)
Settlement 469 days	500	93.6 (±6.14)	6.22 (±0.50)

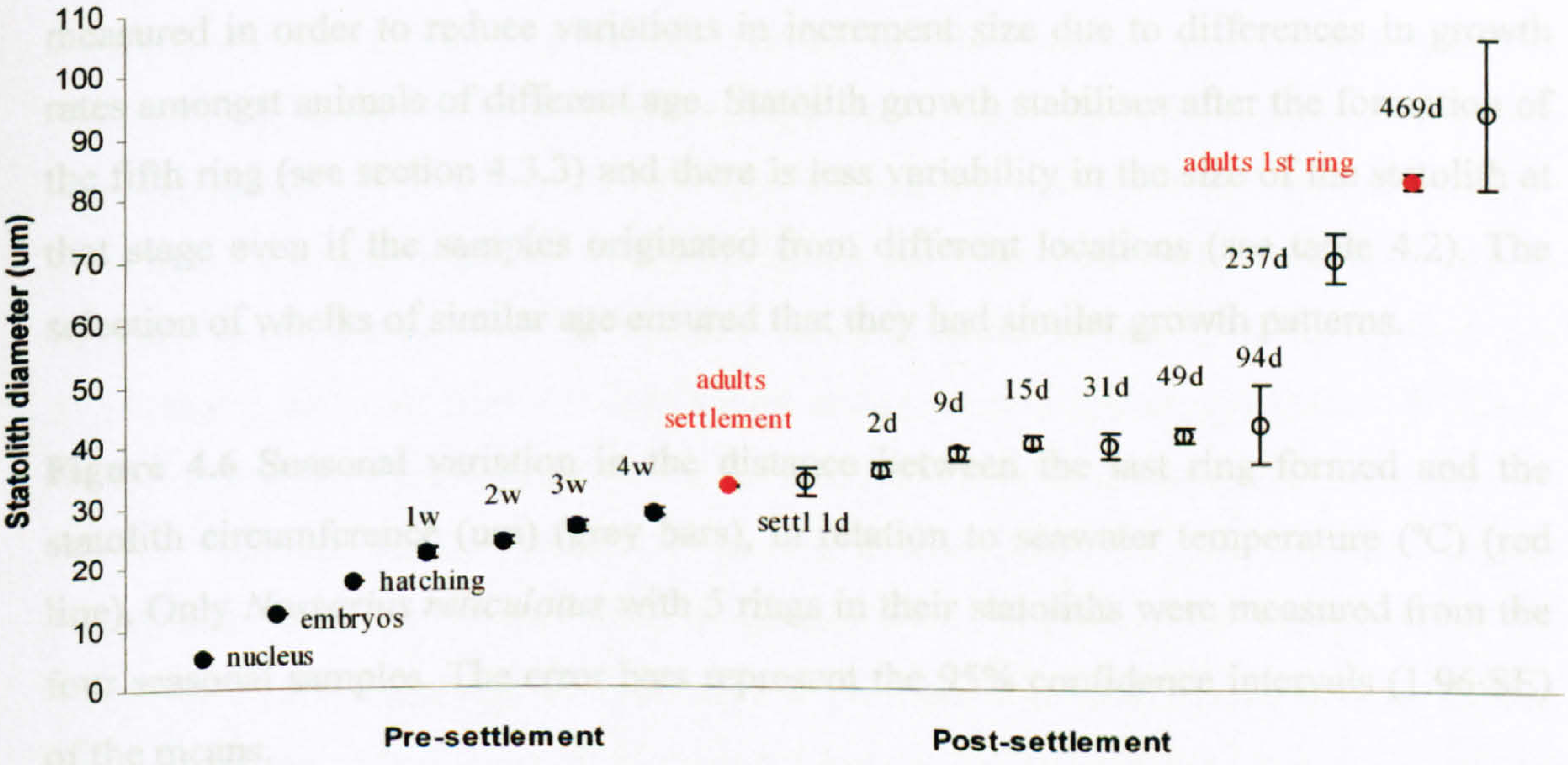
The increase in statolith diameter was studied from the early embryonic stages up to the age of 500 days (469 days from settlement) when the experiment was terminated (table 4.3). The mean statolith diameter of 270-day old juveniles (237 days from settlement) was 70.0 um (±2.00) and the mean statolith diameter of 500-day old juveniles (469 days from settlement) was 93.6 um (±6.14). The mean diameter of the first ring (following the settlement ring) measured in adult statoliths was 82.7 um (±0.55). Since the statolith diameter of whelks <1 year old was 70 um and of whelks >1 year old it was 93.6 um, the formation of a clearly discernible ring on the adult statolith with a diameter of 82.7

Figure 4.4 Larval, juvenile and adult statoliths of *Nassarius reticulatus*: a) the statolith inside the statocyst of a 2-week old larva where no ring has yet been formed, b) the statolith of a 132-day old juvenile (94 days after settlement) in which the nucleus and the settlement ring can be seen, c) the statolith of a 500-day old juvenile (469 days after settlement) where the settlement and the first annual ring can be seen and d) the statolith from an adult whelk in which the settlement ring and 4 annual rings are shown. n: nucleus, sc: statocyst, s:settlement ring, 1-4: annual rings


a
n
sc
50 µm
b
n
S
50 µm
c
n
S
1
50 µm
d
n
S
1
2
3
4
50 µm

um strongly suggests that this ring is deposited after ~1 year and is the first annual ring. The statolith diameter of 270 day old juveniles was significantly smaller than the statolith diameter of 500 days old juveniles, and than the diameter of the first ring on the adult statolith (Anova: F=3.39, p=0.035). However, no significant difference was found between the diameter of the first ring on the adult statolith and the statolith diameter of 500 day old juveniles (2-sample t-test: T=1.69, p=0.092). The first annual ring in the statolith of a juvenile >1 year old is shown in figure 4.4c and its diameter was 95.5 um.

Figure 4.5 Increase in the mean statolith diameter of *Nassarius reticulatus* larvae and juveniles pre- (closed symbols) and post-settlement (open symbols). Labels indicate weeks after hatching and days after settlement. For comparison the mean diameter of the settlement ring and first annual ring measured in the adult statoliths is shown (red symbols). The error bars represent the 95% confidence intervals (1.96·SE) of the means.



From these observations and measurements it is evident that the nucleus of the statolith is formed during embryonic life, the first prominent ring (S) is deposited during settlement of the larvae at the end of their planktonic life, and the following ring (ring 1) is formed after the first year of life is completed (figure 4.5). As indicated before (table 4.3) the statolith diameter of embryos (12.5 um) and newly hatched larvae (17.5 um) was identical in all the individuals examined. Furthermore, no significant difference was found in the diameter of the settlement ring of *Nassarius reticulatus* from different locations (33.2 um) (section 4.3.3). The diameters of the annual rings are, however, significantly different between whelks from different locations. Thus, it can be concluded that for the period between the embryonic and the settlement phase the size of the statolith was not significantly different in animals from different areas. The growth of the statolith is differentiated after the settlement phase when it is under the control of variable environmental factors present in the different locations and ecosystem types.

4.3.5 Seasonality of statolith ring formation

The samples of *Nassarius reticulatus* collected from Rhosneigr during June 2003, August 2003, November 2003 and February 2004 were used in order to investigate possible seasonality in the formation of the growth rings in the statolith. The distance from the last formed dark ring to the circumference of the statolith, which represents the developing growth increment at a particular time, was measured in the statoliths from the 4 seasonal samples (see table 4.4, figure 4.6). Only statoliths with 5 rings were measured in order to reduce variations in increment size due to differences in growth rates amongst animals of different age. Statolith growth stabilises after the formation of the fifth ring (see section 4.3.3) and there is less variability in the size of the statolith at that stage even if the samples originated from different locations (see table 4.2). The selection of whelks of similar age ensured that they had similar growth patterns.

Figure 4.6 Seasonal variation in the distance between the last ring formed and the statolith circumference (um) (grey bars), in relation to seawater temperature (°C) (red line). Only *Nassarius reticulatus* with 5 rings in their statoliths were measured from the four seasonal samples. The error bars represent the 95% confidence intervals (1.96·SE) of the means.

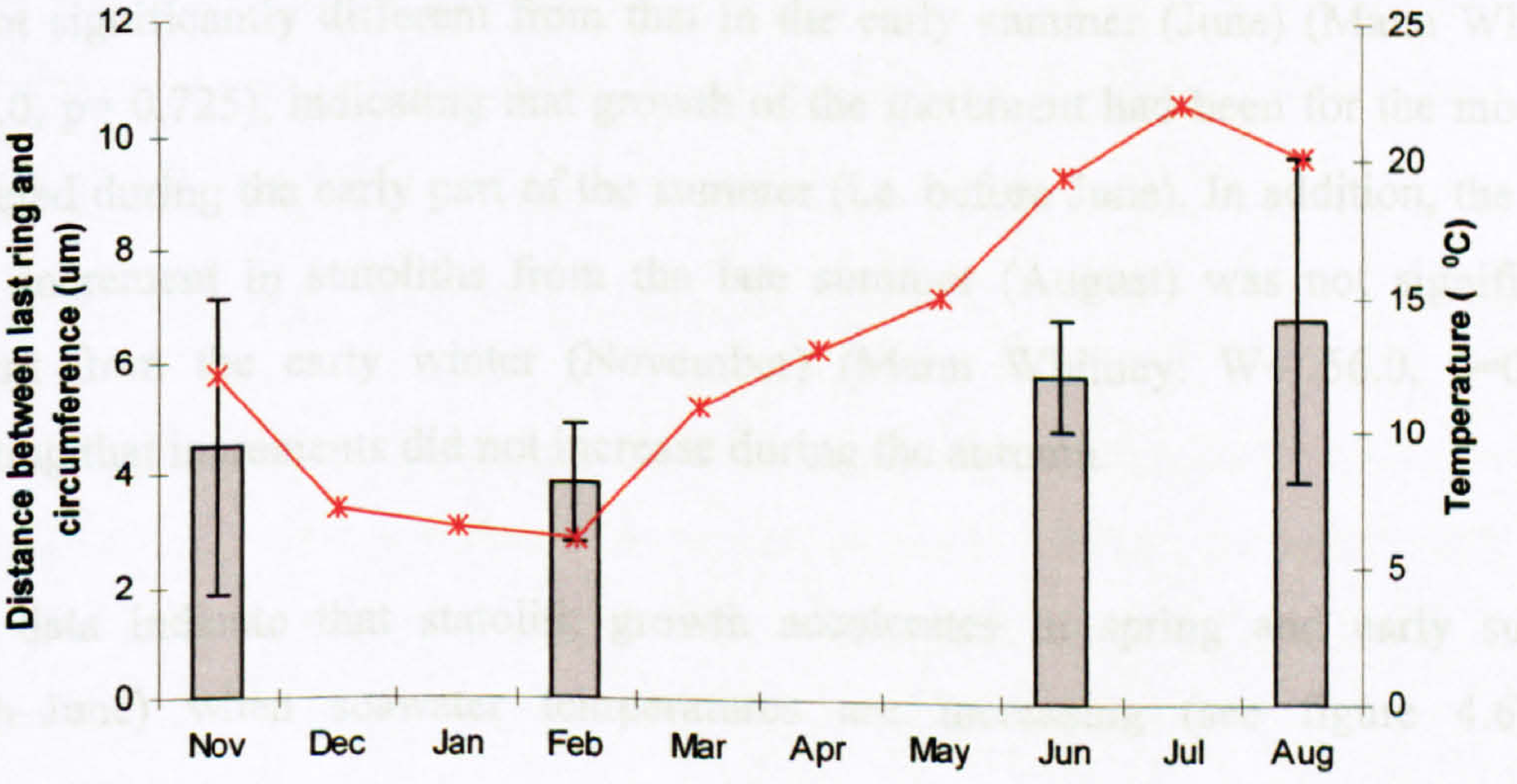


Table 4.4 Mean values (um) (±S.E.) of the distance between the last formed ring and the circumference in the statolith of *Nassarius reticulatus* from the 4 seasonal samples. Only whelks with 5 rings in their statolith were used (N).

Seasonal Sample	N	Mean distance (um)	S.E. (um)
November	7	4.49	1.32
February	10	3.84	0.52
June	11	5.82	0.49
August	6	6.80	1.43

The data from the 4 seasonal samples were not normally distributed (June: A^2= 0.288, p=0.548; August: A^2= 0.738, p=0.025; November: A^2= 0.903, p=0.010; February: A^2= 0.658, p=0.060) and had equal variances (Levene's test= 0.338, p=0.798), so a non-parametric Kruskal-Wallis test was performed. The distance between the last deposited ring and the circumference of the statolith was significantly different between the 4 seasonal samples (H=8.83, p=0.032). Further detailed comparisons between pairs of samples were conducted in order to investigate in which months the measurements were significantly different. When the data were normal and had equal variances a 2-sample t-test was used, and when the data were non-normal but had equal variances a Mann-Whitney test was used. The early winter statolith measurements (November) were not significantly different from the late winter measurements (February) (Mann Whitney: W=64.0, p=0.961), indicating that the growth increment at the statolith circumference did not increase during winter. The growth increment in the early summer sample (June) was significantly larger than in the late winter (February), (2 sample t-test: T= 2.79, p=0.012), indicating that the highest percentage of the increment was formed during early summer. The width of the increment in the late summer (August), however, was not significantly different from that in the early summer (June) (Mann Whitney: W=95.0, p= 0.725), indicating that growth of the increment had been for the most part completed during the early part of the summer (i.e. before June). In addition, the width of the increment in statoliths from the late summer (August) was not significantly different from the early winter (November) (Mann Whitney: W= 56.0, p=0.054), indicating that increments did not increase during the autumn.

These data indicate that statolith growth accelerates in spring and early summer (March–June) when seawater temperatures are increasing (see figure 4.6) and assimilated energy is mostly allocated to statolith and shell growth. Between June and August statolith growth slows down because more energy is used for reproductive activity such as egg laying (see chapter 5). During the autumn and winter, when the seawater temperature decreases, growth rates are low (see chapter 3) and the statoliths do not increase in size. Consequently, formation of the light wider ring in the statolith occurs between spring and early summer when seawater temperatures are rising and more energy is allocated to growth. Formation of the dark narrow ring on the statolith occurs between late summer and winter when growth slows down, probably due to reproductive activity initially, and in response to declining seawater temperatures later.

4.3.6 The relationship between statolith diameter and shell length of adult *Nassarius reticulatus*

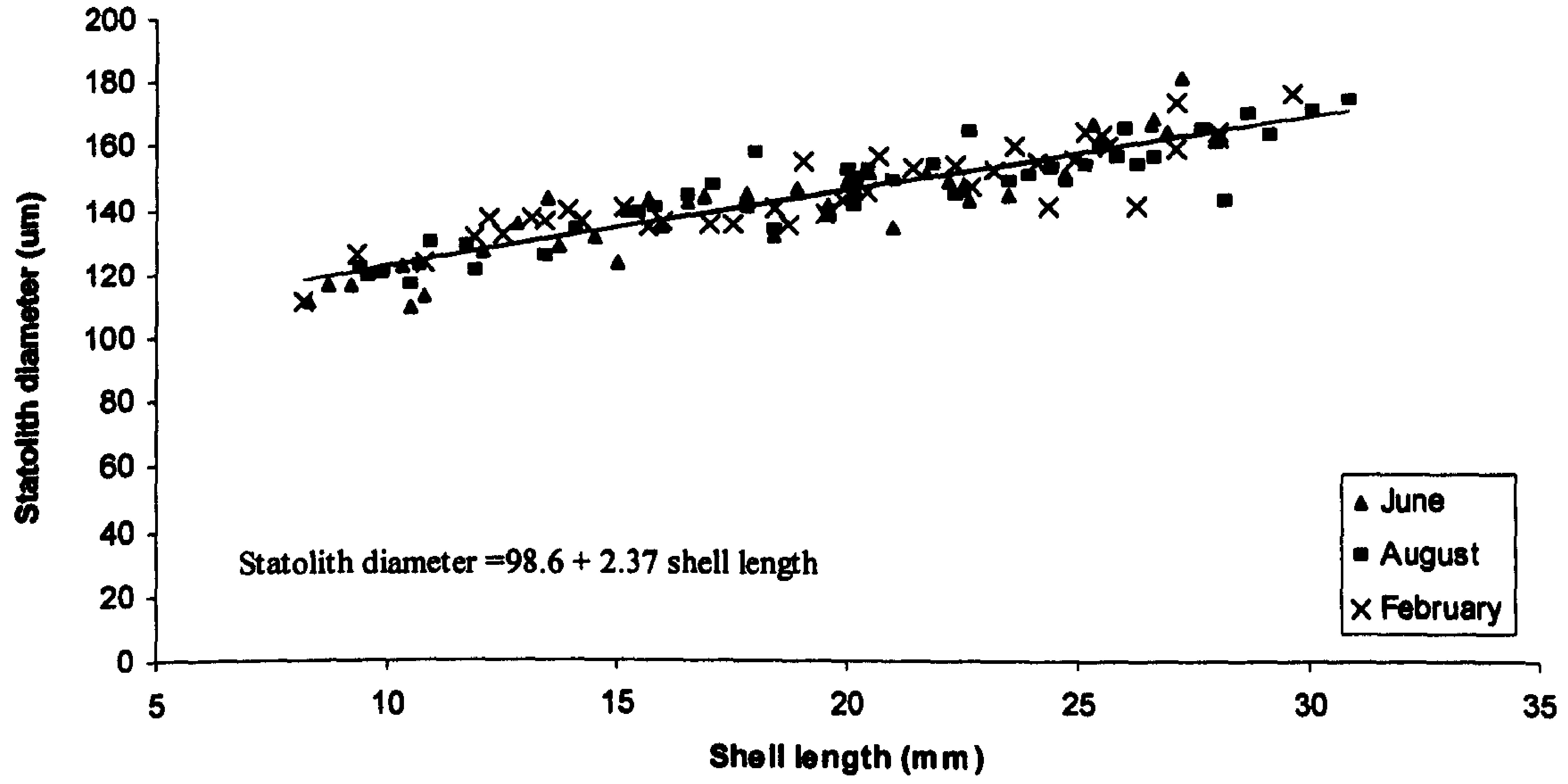
4.3.6.1 Comparison between seasonal samples

A significant correlation and a significant linear regression were found between statolith diameter and shell length for each of three seasonal samples collected from the Rhosneigr lagoon in June 2003 ($r=0.911$, $p<0.001$; $F= 184.07$, $p<0.001$), August 2003 ($r= 0.910$, $p<0.001$; $F=180.81$, $p<0.001$) and February 2004 ($r=0.890$, $p<0.001$; $F=140.93$, $p<0.001$). A General Linear Model (GLM) was used in order to compare the relationship between statolith diameter and shell length of the three seasonal samples. The residuals in all samples were normally distributed (June: $A^2= 0.270$, $p=0.660$, August: $A^2= 0.467$, $p=0.238$, February: $A^2= 0.295$, $p=0.579$) and the data had equal variances (Bartlett's test $=0.266$, $p=0.876$). No significant difference was detected between the three seasonal samples concerning the intercept ($F=2.27$, $p=0.108$) or the slope ($F=1.99$, $p=0.142$) of the regression. The regression for the three seasonal samples grouped as one intertidal lagoon sample was significant ($F=506.83$, $p<0.001$) and can be described by the equation:

$$\text{Statolith Diameter (um)} = 98.6 + 2.37 \cdot \text{shell length (mm)} \qquad \text{(equation 1)}$$

The above equation could then be used to estimate the shell length from measurements of statolith diameter for any *Nassarius reticulatus* collected from the lagoon area in any month and is applicable to a range of shell lengths between 8.2 mm and 30.8 mm (figure 4.7).

Figure 4.7 The relationship between statolith diameter (um) and shell length (mm) for a range of different sized *Nassarius reticulatus* (8.2-30.8 mm) collected from the Rhosneigr intertidal lagoon during June 2003, August 2003 and February 2004.



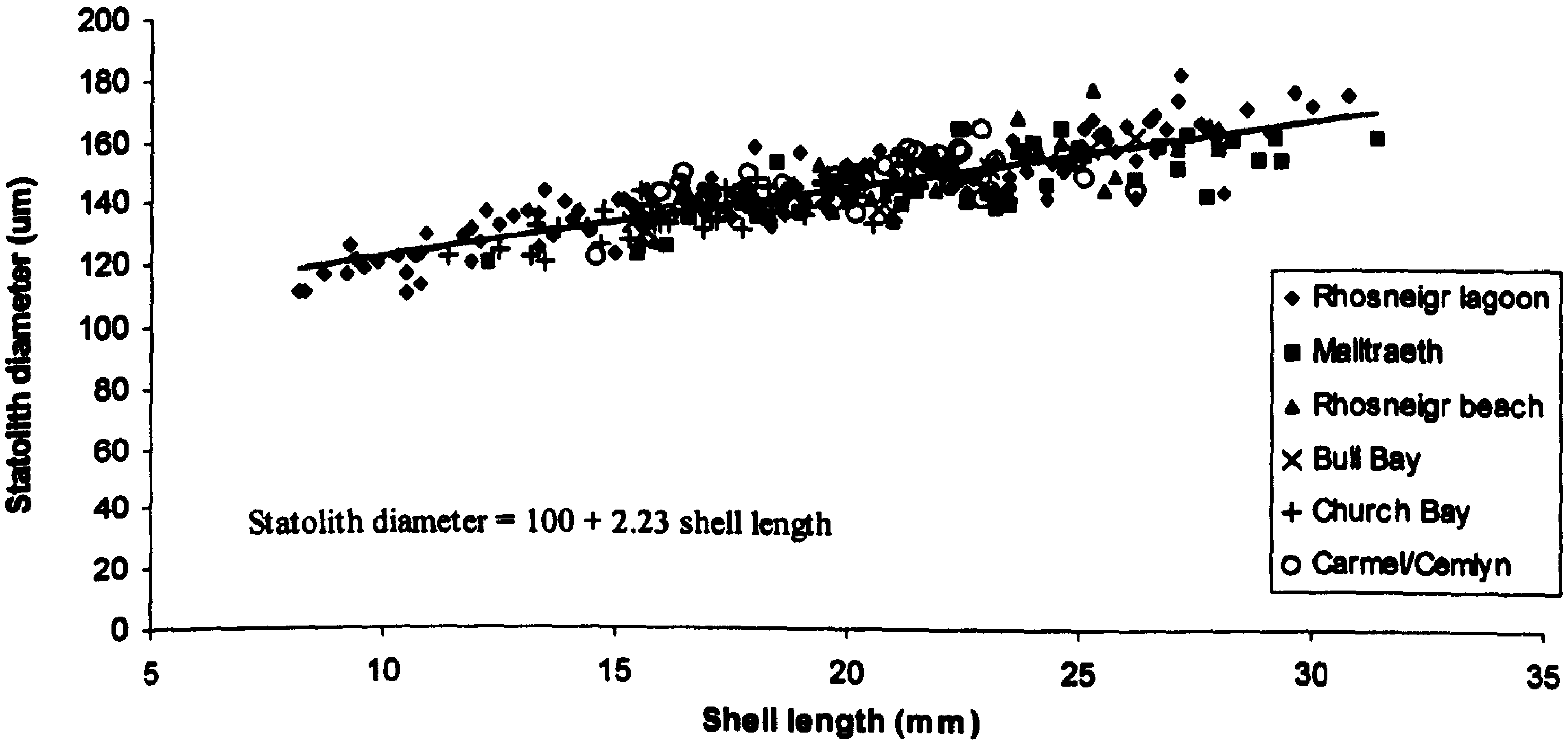
4.3.6.2 Comparison between regional samples

Since there was no significant difference between the three seasonal samples from the lagoon area these data were pooled to form one lagoon sample. A significant correlation was found between statolith diameter and shell length for each of the six regional samples (Rhosneigr lagoon r=0.902, p<0.001; Malltraeth r= 0.755, p<0.001; Rhosneigr beach r=0.729, p<0.001; Bull Bay r= 0.891, p<0.001; Church Bay r= 0.776, p<0.001; Carmel Head-Cemlyn Bay r= 0.553, p<0.001). A significant linear regression was found between statolith diameter and shell length for the Rhosneigr lagoon (F=506.83, p<0.001), Malltraeth (F= 50.33, p<0.001), Rhosneigr beach (F= 43.13, p<0.001), Bull Bay (F= 42.51, p<0.001), Church Bay (F= 57.68, p<0.001), and the Carmel Head-Cemlyn Bay (F=14.08, p<0.001). In order to investigate possible differences in the relationship between statolith diameter and shell length from the different areas a General Linear Model (GLM) analysis was performed. The residuals in all samples were normally distributed (Rhosneigr lagoon: A^2=0.500, p=0.205; Malltraeth: A^2= 0.212, p=0.844; Rhosneigr beach: A^2= 0.381, p=0.386; Bull Bay: A^2= 0.574, p=0.110; Church Bay: A^2= 0.183, p=0.905; Carmel Head-Cemlyn Bay: A^2= 0.635, p=0.090) and the data had equal variances (Bartlett's= 10.099, p=0.072). No significant difference was found between the six areas in the intercept (F=1.24, p=0.293) or the slope (F=1.53, p=0.180) of the regression. The regression for the six regional samples was significant (F=726.01, p<0.001) and can be described by the equation:

Statolith Diameter (um) = 100 + 2.23 · shell length (mm) (equation 2)

The above equation could then be used to estimate shell length from measurements of statolith diameter for *Nassarius reticulatus* collected from any of the sampling sites around Anglesey and it is applicable to a range of shell lengths between 8.2 mm and 31.4 mm (figure 4.8).

Figure 4.8 The relationship between statolith diameter (um) and shell length (mm) for a range of different sized *Nassarius reticulatus* (8.2-31.4 mm) collected from Rhosneigr lagoon, Rhosneigr beach, Malltraeth, Bull Bay, Church Bay and Carmel Head to Cemlyn Bay.



4.3.6.3 Comparison between males, females and imposex affected females

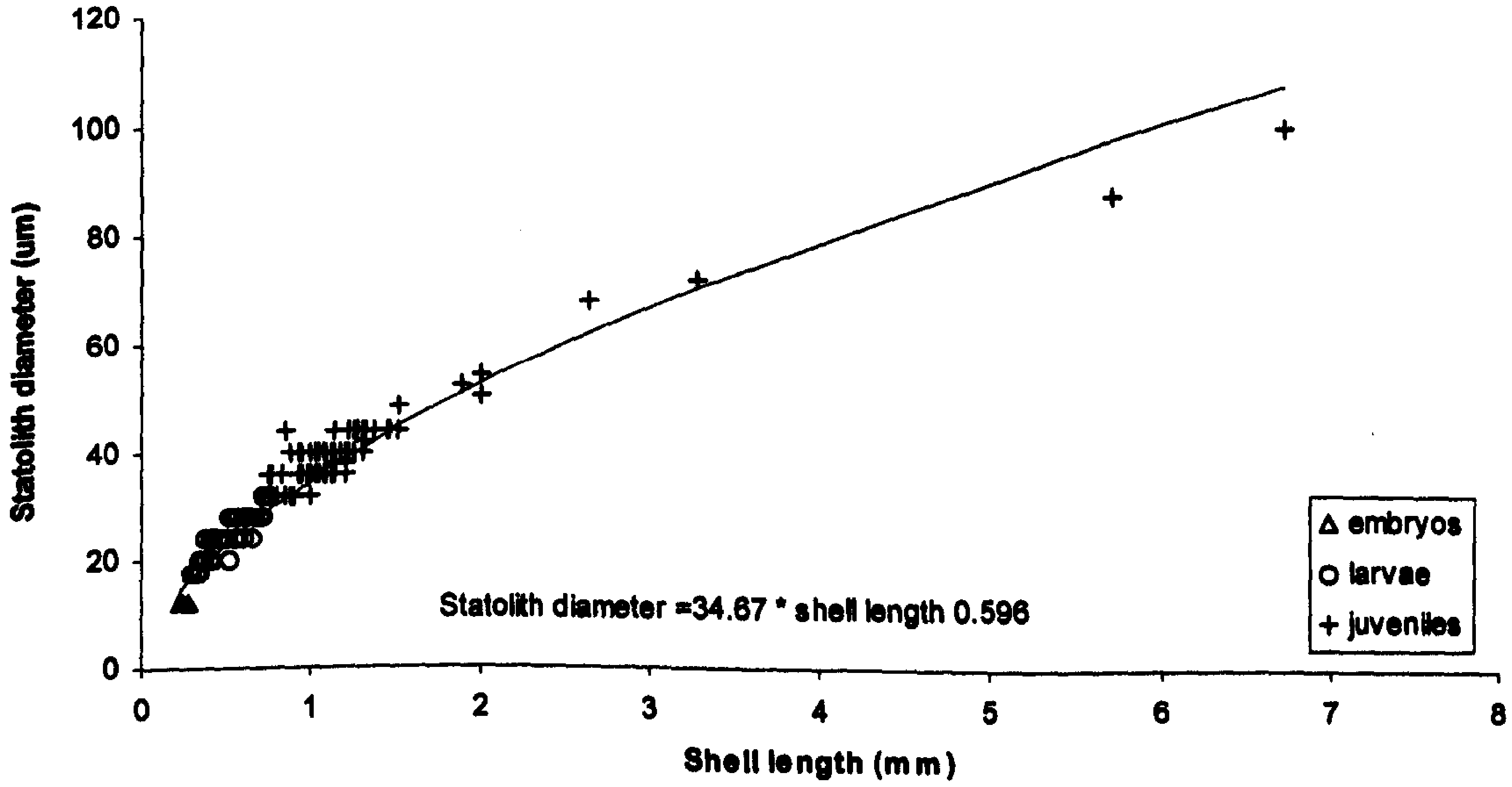
In the two previous sections (4.3.6.1 and 4.3.6.2) it was shown that there was no significant difference in the relationship between statolith diameter and shell length of *Nassarius reticulatus* that were collected during the different seasons or from different areas around Anglesey. This allows grouping of the whelks from all samples (seasonal and regional) in order to investigate if there is a significant difference in the relationship between statolith diameter and shell length regarding animals of different sex or imposex stage. A significant correlation and regression was found between statolith diameter and shell length of female, male and imposex affected *N. reticulatus* (Females:

r=0.819, p<0.001; F=332.3, p<0.001, Males: r=0.644, p<0.001; F=31.19, p<0.001, Imposex: r=0.779, p<0.001; F=90.87, p<0.001). All the data were normally distributed (Females: A^2=0.459, p=0.259; Males: A^2=0.276, p=0.643; Imposex: A^2=0.290, p=0.601) and had equal variances (Bartlett's T=4.327, p=0.115). A GLM analysis indicated that there were no significant difference between males, females and imposex affected whelks, regarding the intercept (F=0.54, p=0.583) or the slope (F=0.46, p=0.635) of the regression. In conclusion, equation (2) presented in section 4.3.6.2 can be used to estimate shell length from measurements of the statolith diameter of female, male and imposex affected *N. reticulatus* collected during any season, from any of the sampling sites around Anglesey and it is applicable to a wide range of shell sizes (8.2 to 31.4 mm).

4.3.7 The relationship between statolith diameter and shell length of embryos, larvae and juvenile *Nassarius reticulatus*

Samples of 10 *Nassarius reticulatus* embryos, 100 larvae (age 1-28 days) and 115 juveniles (age 23-500 days) were used to establish the relationship between statolith diameter and shell length (figure 4.9). A significant correlation (r=0.957, p<0.001) was found between statolith diameter and shell length. Analysis of variance presented a significant probability for the regression (F=184.44, p<0.001) and the data were normally distributed (A^2= 0.317, p=0.513). However, a strong pattern was observed in

Figure 4.9 The relationship between statolith diameter (um) and shell length (mm) for embryos, larvae and juveniles of *Nassarius reticulatus* (size range 0.2-6.7 mm).



the plot of the residuals versus fits indicating that the data could not meet the criteria for a linear regression. After a log transformation was applied on the statolith diameter and the shell length data, a significant correlation and regression were found ($r=0.991$, $p<0.001$; $F=952.49$, $p<0.001$), the data were normal ($A^2= 0.703$, $p=0.055$), and there was no obvious pattern in the plot of residuals versus fits. The exponential relationship between statolith diameter and shell length of larvae and juveniles is described by the following equation:

$$\text{Statolith Diameter (um)} = 34.67\cdot \text{shell length (mm)}^{0.596} \qquad \text{(equation 3)}$$

4.3.8 The relationship between statolith diameter and shell length of adults, larvae and juvenile *Nassarius reticulatus*

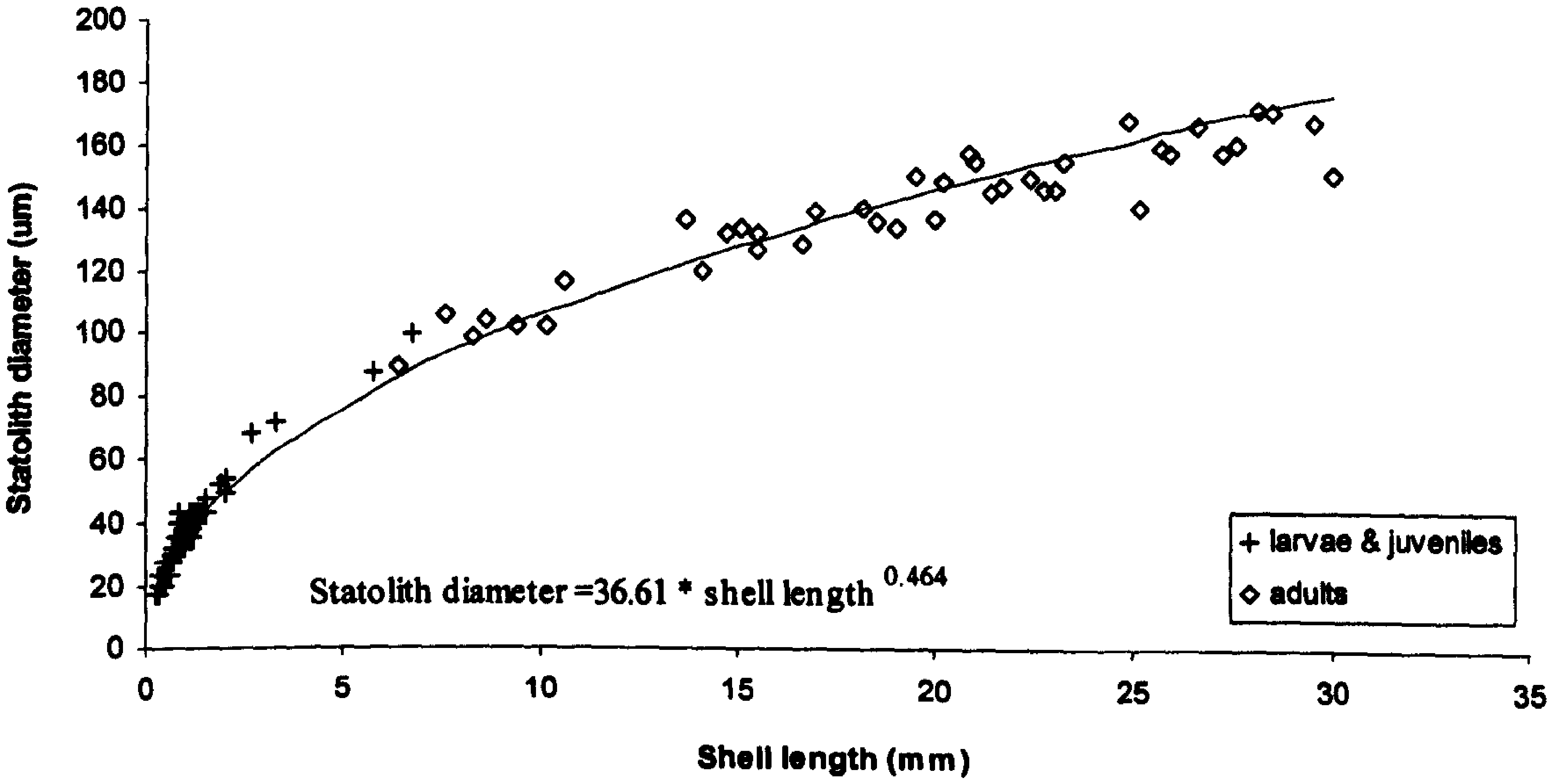
In the previous sections (4.3.6.2 and 4.3.7) two equations describing the relationship between statolith diameter and shell length were obtained: a linear equation (equation 2) for all the adults ranging in size from 8.2 to 31.4 mm and an exponential equation (equation 3) for embryos, larvae and juveniles ranging in size from 0.2 to 6.7 mm. In order to estimate a relationship containing a wide range of sizes encompassing larvae and adults, a sample containing also some smaller animals (size range 6.4-30 mm) was used. The statolith data from larvae and juveniles (section 4.3.7) were used (size range from 0.3 to 6.7 mm) but the embryo data were not included in the analysis due to the high variability they added to the regression.

A strong correlation was found between statolith diameter and shell length ($r=0.952$, $p<0.001$) and the regression between these two variables was significant ($F=552.32$, $p<0.001$). Although the data were normally distributed ($A^2= 0.421$, $p=0.313$), the strong pattern of the plot of residuals versus fits indicated that the relationship was non- linear. After performing a log transformation on the data, statolith diameter and shell length were strongly correlated ($r=0.993$, $p<0.001$) and the regression was significant ($F=3886.32$, $p<0.001$). The data were normally distributed ($A^2= 0.568$, $p=0.135$) and there was no obvious pattern in the plot of residuals versus fits, indicating that the log transformed data met all the criteria for the regression analysis. The relationship between statolith diameter and shell length of adults, juveniles and larvae was exponential and is described by the equation:

Statolith Diameter (um) = 36.31· shell length (mm) $^{0.464}$ (equation 4)

The above exponential equation could then be used to estimate the shell length from the measurements of statolith diameter for all life stages of *Nassarius reticulatus*, (adults, juveniles and larvae), and it is applicable to a range of shell lengths from 0.3 mm up to 30 mm (figure 4.10).

Figure 4.10 The relationship between statolith diameter (um) and shell length (mm) for adults, juveniles and larvae of *Nassarius reticulatus* of a shell size range from 0.3 mm to 30 mm.



4.3.9 Estimation of length-at-age curves

The exponential equation (4) is applicable to a range of sizes (0.3-30 mm) of male, female or imposex affected whelks collected from around Anglesey, and was used to estimate the shell length of *Nassarius reticulatus* during the formation of each annual ring (length-at-age data). Since larvae of *N. reticulatus* settle 25-51 days after hatching (see chapter 6), the shell length at formation of the settlement ring was calculated for an average age of 38 days (0.10 year). Von Bertalanffy growth curves have been fitted to these length-at-age data using the equation $L(t) = L_\infty (1-e^{-K(t-t_0)})$, where L_∞ is the asymptotic maximum shell length, K is the growth constant and t_0 is the theoretical age at which shell length would have been zero. The VBG growth constants were estimated using the FISAT II program (version 1.1.3).

Any differences between the VBG growth curves were tested by comparing the von Bertalanffy growth parameters L_∞, K and t_0 of each group (i.e. different sexes, different sampling areas) in pairs using Hotelling's T^2 test, modified to an F statistic (Bernard, 1981). The critical value of T_0^2, which is a modified tabulated F statistic, is used to test the estimated T^2 statistic for significance (a=0.001). When $T^2 > T_0^2$ growth between a pair of groups is described as dissimilar, and when $T^2 < T_0^2$ growth is described as similar. When Hotelling's T^2 test was found to be significant ($T^2 > T_0^2$) the Roy-Bose simultaneous confidence intervals around differences between the parameters were estimated in order to indicate which of the three parameters contributed most to the statistical significance (see Morrison, 1976). When the confidence intervals did not include zero the parameters had a significant influence on the differences in growth between the two comparisons.

4.3.9.1 Comparison of growth between male and female *Nassarius reticulatus*

Growth patterns of female, male and imposex affected whelks were studied in order to investigate possible differences in growth between the sexes, as well as the effect of imposex on growth. Equation (4) was used to estimate the shell length of *Nassarius reticulatus* during the formation of each annual ring and a VBG curve fitted to the length-at-age data. The VBG growth constants for females, males and imposex affected animals from Rhosneigr beach, Church Bay, Bull Bay and Carmel Head-Cemlyn Bay areas were estimated (table 4.5) and are plotted in figure 4.11. Rhosneigr lagoon and Malltraeth were not analysed due to the low numbers of male *N. reticulatus* collected. Comparisons of the VBG growth constants between normal (non-affected by TBT) females and males, and between normal females and imposex affected females from each area were performed using Hotelling's T^2 test (table 4.6). No significant differences were found between the growth pattern of females and males or between females and imposex affected gastropods from the 4 areas. Therefore estimates of Roy-Bose confidence intervals were not required.

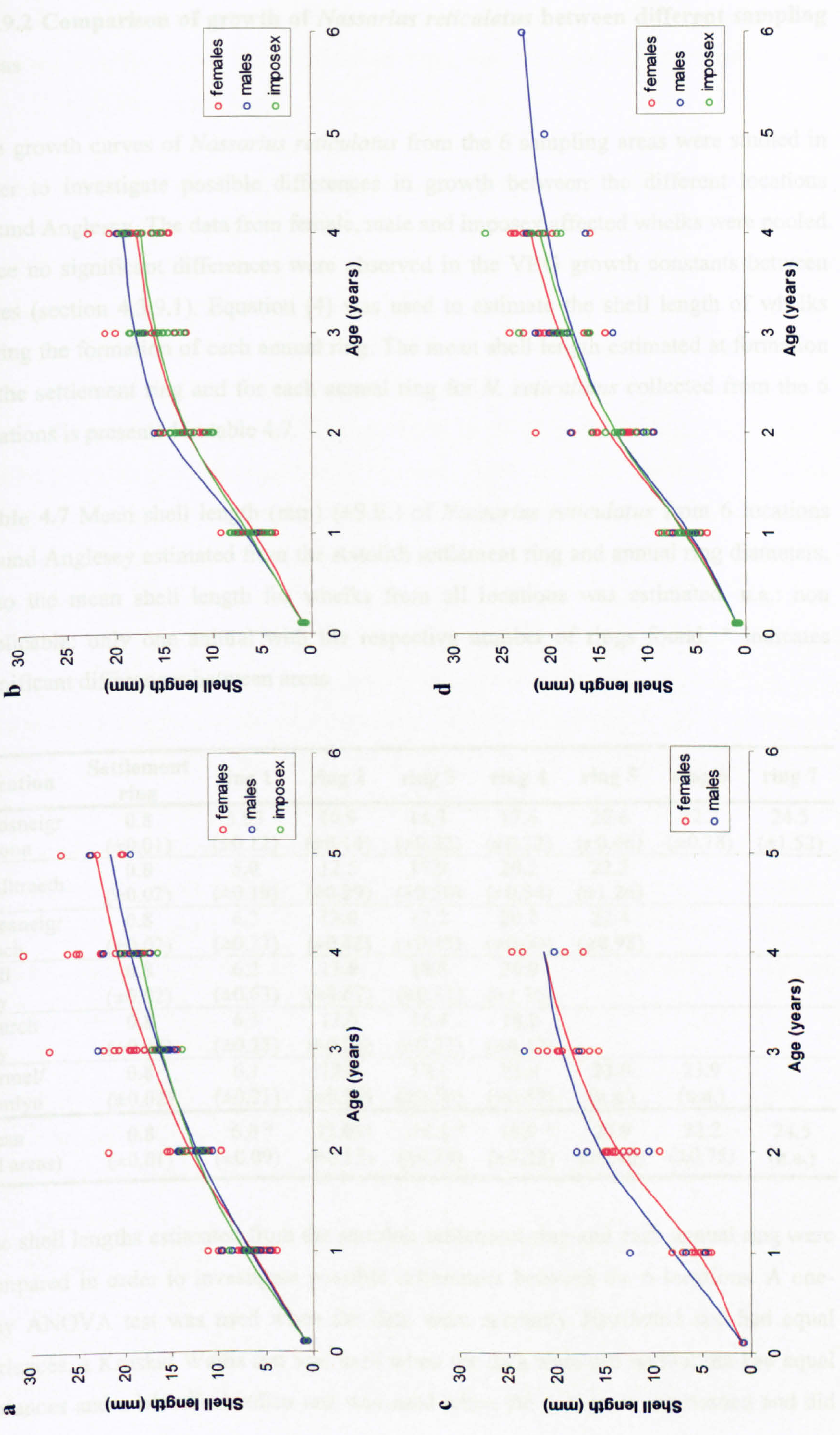
Table 4.5 Growth constants (L_∞, K and t_0) (±S.E.) in the von Bertalanffy growth curves of female, male and imposex affected *Nassarius reticulatus* from Rhosneigr beach, Church Bay, Bull Bay and the Carmel Head-Cemlyn Bay area. The size of each sample (N) is indicated. n.a. not applicable, only one imposex affected female was found at Bull Bay, thus a VBG curve was not constructed.

	L_∞ (mm)	K	t_0 (y)	N
Rhosneigr beach				
Females	27.8 (±3.18)	0.37 (±0.10)	0.27 (±0.14)	26
Males	27.1 (±5.47)	0.32 (±0.14)	0.10 (±0.25)	10
Imposex	22.7 (±4.46)	0.42 (±0.21)	0.06 (±0.33)	4
Church Bay				
Females	19.6 (±1.41)	0.71 (±0.16)	0.49 (±0.11)	18
Males	20.3 (±1.10)	0.90 (±0.21)	0.54 (±0.13)	4
Imposex	19.3 (±1.60)	0.66 (±0.17)	0.38 (±0.13)	13
Bull Bay				
Females	25.5 (±3.86)	0.52 (±0.17)	0.54 (±0.13)	8
Males	23.1 (±9.66)	0.70 (±1.00)	0.40 (±0.65)	2
Imposex	n.a	n.a	n.a	0
Carmel Head - Cemlyn Bay				
Females	27.0 (±3.56)	0.48 (±0.15)	0.47 (±0.14)	13
Males	24.3 (±3.27)	0.52 (±0.20)	0.48 (±0.20)	5
Imposex	27.8 (±5.33)	0.39 (±0.18)	0.34 (±0.19)	11

Table 4.6 Hotelling's T^2 test for comparison of growth parameters between females and males, and between females and imposex affected females *Nassarius reticulatus*. n.a. not applicable, only one imposex affected female was found at Bull Bay, thus a VBG curve was not constructed.

	T^2	T_0^2	Comparison
Rhosneigr beach			
Females - Males	6.51	22.63	$T^2<T_0^2$ similar
Females-Imposex	8.16	24.23	$T^2<T_0^2$ similar
Church Bay			
Females - Males	2.83	28.33	$T^2<T_0^2$ similar
Females-Imposex	11.68	24.17	$T^2<T_0^2$ similar
Bull Bay			
Females - Males	8.92	94.80	$T^2<T_0^2$ similar
Females-Imposex	n.a.	n.a.	n.a.
Carmel Head– Cemlyn Bay			
Females - Males	10.08	33.26	$T^2<T_0^2$ similar
Females-Imposex	26.34	26.73	$T^2<T_0^2$ similar

Figure 4.11 Von Bertalanffy growth curves fitted to the length-at-age data for *Nassarius reticulatus*. Estimates of shell length were calculated from the statolith diameter of each annual ring for females (red symbols), males (blue symbols) and imposex affected females (green symbols) collected from a) Rhosneigr beach, b) Church Bay, c) Bull Bay and d) Carmel to Cemlyn Bay area. Only one imposex affected female was found at Bull Bay, thus a VBG curve could not be constructed.


a
b
c
d
Shell length (mm)
Age (years)
0
5
10
15
20
25
30
0
1
2
3
4
5
6
females
males
imposex

4.3.9.2 Comparison of growth of *Nassarius reticulatus* between different sampling areas

The growth curves of *Nassarius reticulatus* from the 6 sampling areas were studied in order to investigate possible differences in growth between the different locations around Anglesey. The data from female, male and imposex affected whelks were pooled since no significant differences were observed in the VBG growth constants between sexes (section 4.3.9.1). Equation (4) was used to estimate the shell length of whelks during the formation of each annual ring. The mean shell length estimated at formation of the settlement ring and for each annual ring for *N. reticulatus* collected from the 6 locations is presented in table 4.7.

Table 4.7 Mean shell length (mm) (±S.E.) of *Nassarius reticulatus* from 6 locations around Anglesey estimated from the statolith settlement ring and annual ring diameters. Also the mean shell length for whelks from all locations was estimated. n.a.: non applicable: only one animal with the respective number of rings found. * indicates significant differences between areas

Location	**Settlement ring**	**ring 1**	**ring 2**	**ring 3**	**ring 4**	**ring 5**	**ring 6**	**ring 7**
Rhosneigr lagoon	0.8 (±0.01)	5.93 (±0.12)	10.9 (±0.14)	14.3 (±0.22)	17.6 (±0.32)	20.6 (±0.46)	22.2 (±0.78)	24.5 (±1.52)
Malltraeth	0.8 (±0.02)	5.0 (±0.19)	12.5 (±0.29)	17.9 (±0.50)	20.2 (±0.54)	22.2 (±1.26)		
Rhosneigr beach	0.8 (±0.02)	6.7 (±0.27)	13.0 (±0.32)	17.2 (±0.43)	20.2 (±0.50)	22.4 (±0.98)		
Bull Bay	0.8 (±0.02)	6.2 (±0.63)	13.6 (±0.67)	18.8 (±0.73)	20.9 (±1.35)			
Church Bay	0.8 (±0.02)	6.3 (±0.25)	13.0 (±0.26)	16.4 (±0.37)	18.2 (±0.42)			
Carmel/ Cemlyn	0.8 (±0.02)	6.1 (±0.21)	13.4 (±0.50)	19.1 (±0.50)	21.4 (±0.55)	23.0 (n.a.)	23.9 (n.a.)	
Mean (all areas)	0.8 (±0.01)	6.0 * (±0.09)	12.01* (±0.13)	16.3 * (±0.19)	19.0 * (±0.22)	20.9 (±0.40)	22.2 (±0.75)	24.5 (n.a.)

The shell lengths estimated from the statolith settlement ring and each annual ring were compared in order to investigate possible differences between the 6 locations. A one-way ANOVA test was used when the data were normally distributed and had equal variances, a Kruskal Wallis test was used when the data were not normal but had equal variances and a Mood's Median test was used when the data were not normal and did not have equal variances. Comparisons were made only up to the fifth ring; only whelks collected from the lagoon had formed more rings, and only one individual from Carmel Head-Cemlyn Bay had 6 rings.

The estimated mean shell length during the formation of the settlement ring was 0.8 mm and there was no significant difference between the 6 locations (Kruskal Wallis: H=0.64, p=0.986). The estimated mean shell length at settlement (0.8 mm) was similar with the mean shell length measured in recently settled larvae (0.78 mm) (see table 4.3). However, the shell lengths estimated from the first, second, third and fourth annual rings were significantly different between the 6 locations (first ring: Mood's Median x^2=23.59, p<0.001; second ring: Kruskal Wallis H=74.65, p<0.001; third ring: Kruskal Wallis H=95.76, p<0.001; fourth ring: Kruskal Wallis H=42.01, p<0.001). The mean shell length at formation of the first ring ranged from 5.0 mm in Malltraeth to 6.7 mm in Rhosneigr beach, at the second ring from 10.9 mm in the Rhosneigr lagoon area to 13.6 mm in the Bull Bay area, at the third ring from 14.3 mm in the Rhosneigr lagoon area to 19.1 mm in the Carmel Head-Cemlyn Bay area, and at the fourth ring from 17.6 mm in the Rhosneigr lagoon to 21.4 mm in the Carmel Head-Cemlyn Bay area (see table 4.7). Snails with 5 rings were only found at Rhosneigr lagoon and beach and at Malltraeth, and only one individual was found in the Carmel Head-Cemlyn area. No significant differences were found in the shell length (20.9 mm) during the formation of the fifth ring between these 4 areas (Anova F=1.31, p=0.278). The above comparisons indicate that there is no variation in the size of the shell at settlement even if the gastropods develop and settle in different habitats. However, the effect of different environmental factors (wave exposure, food availability) during benthic adult life at different locations results in significant variations in shell growth during the first 4 years of life.

In order to investigate thoroughly the growth patterns of *Nassarius reticulatus* from the 6 different locations, von Bertalanffy growth curves were fitted to the length-at-age data estimated from the statolith rings using the FISAT II program (version 1.1.3). The estimated growth constants (L_∞, K and t_0) for the gastropods from each area are shown in table 4.8. The length-at-age data and the fitted von Bertalanffy curves are presented in figure 4.12 for each area and are summarised in figure 4.13 for all areas. Differences between the growth curves of *N. reticulatus* from the 6 sampling areas were investigated by comparing pairs of von Bertalanffy growth constants (L_∞, K and t_0)

from each area using Hotelling's T^2 test. When significant Hotelling's T^2 tests were found ($T^2 > T_0^2$), Roy-Bose simultaneous confidence intervals around differences between the VBG constants were estimated in order to indicate which of the three constants contributed most to the statistical significance (table 4.9).

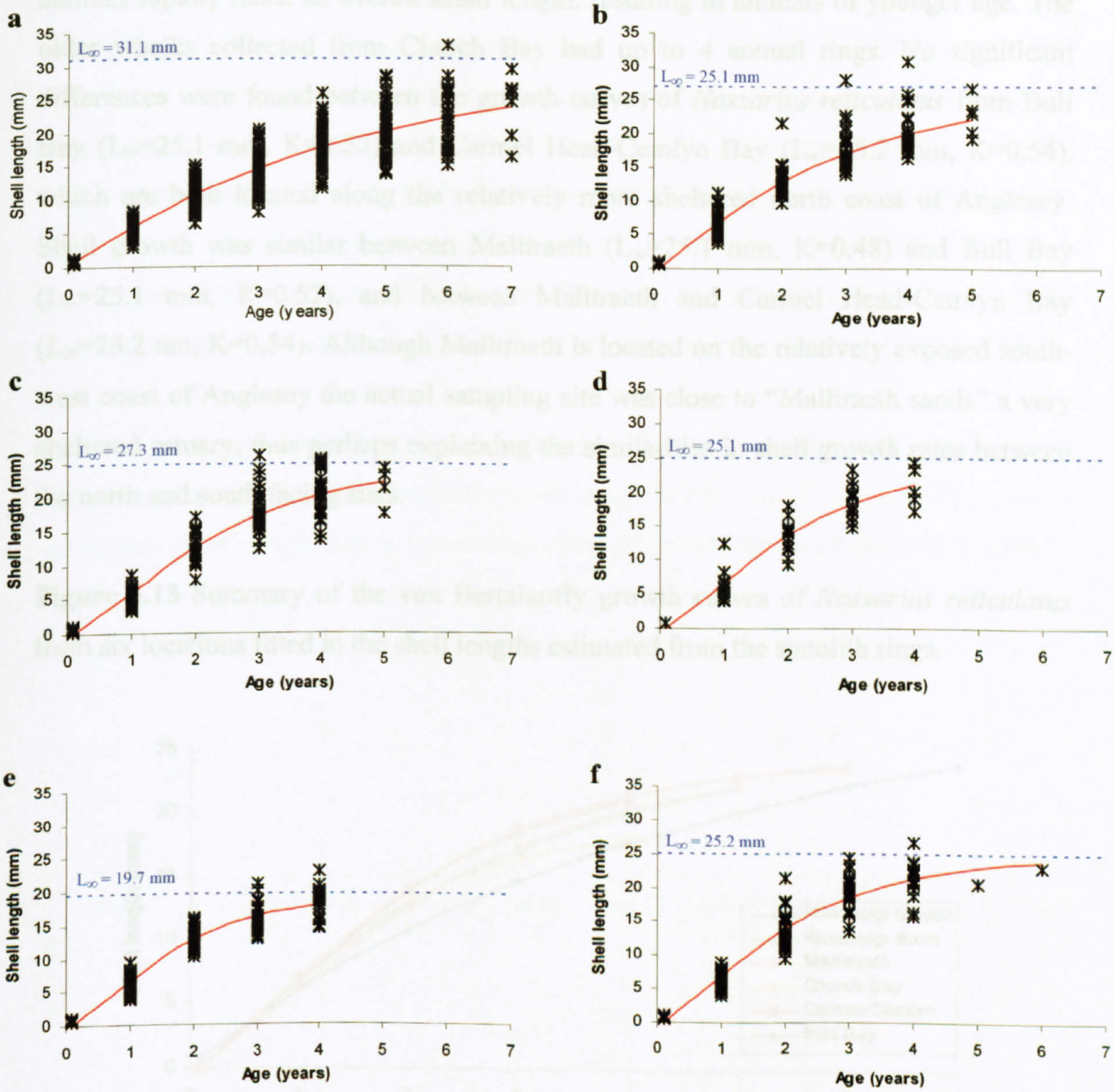
Table 4.8 Growth constants (L_∞, K and t_0) (±S.E.) in the von Bertalanffy curves describing the patterns of *Nassarius reticulatus* shell growth from 6 different locations. The size of each population (N) is indicated.

Location	L_∞ (mm)	K	t_0 (y)	N
Rhosneigr lagoon	31.1 (±2.63)	0.21 (±0.03)	-0.02 (±0.09)	106
Malltraeth	25.1 (±1.95)	0.48 (±0.10)	0.55 (±0.07)	40
Rhosneigr beach	27.3 (±4.85)	0.35 (±0.13)	0.20 (±0.14)	40
Bull Bay	25.1 (±2.35)	0.52 (±0.10)	0.46 (±0.07)	12
Church Bay	19.7 (±1.89)	0.69 (±0.17)	0.45 (±0.10)	36
Carmel/Cemlyn	25.2 (±1.82)	0.54 (±0.09)	0.50 (±0.08)	29

Table 4.9 Hotelling's T^2 test for comparison of pairs of VBG growth constants between sampling areas and Roy-Bose confidence intervals (C.I.) for each growth parameter. † indicates significant differences in shell growth between pairs of areas ($T^2 > T_0^2$), * indicates which parameter contributes most to the differences in shell growth (C.I. does not includes zero), n.a. non-applicable estimation of the Roy-Bose CI when the growth constants are similar.

Pairs of areas compared	T^2	T_0^2	C.I. for L_∞	C.I. for K	C.I. for t_0
Lagoon-Rhosneigr shore †	270.67	17.65	4.61; 3.05 *	0.64; -0.92	0.56; -0.99
Lagoon- Malltraeth †	1327.81	17.65	6.78; 5.22 *	0.51; -1.05	0.21; -1.35
Lagoon – Church Bay †	1467.66	17.65	12.24; 10.62 *	0.33; -1.29	0.34; -1.28
Lagoon – Carmel/Cemlyn †	959.10	17.67	6.82; 5.06 *	0.55; -1.21	0.36; -1.40
Lagoon – Bull Bay †	390.46	17.71	7.29; 4.73 *	0.97; -1.59	0.80; -1.76
Malltraeth – Rhosneigr †	211.80	21.86	-1.13; -3.22 *	1.18; -0.92	1.40; -0.70
Malltraeth – Church Bay †	64.64	21.89	6.50; 4.36 *	0.87; -1.29	1.18; -0.98
Malltraeth – Carmel/Cemlyn	4.22	21.96	n.a	n.a	n.a
Malltraeth – Bull Bay	8.95	22.19	n.a	n.a	n.a
Rhosneigr shore – Church Bay †	309.32	21.89	8.68; 6.53 *	0.74; -1.42	0.83; -1.33
Rhosneigr shore – Carmel/Cemlyn †	140.55	21.96	3.25; 0.97 *	0.95; -1.33	0.84; -1.44
Rhosneigr shore – Bull Bay †	53.01	22.19	3.73; 0.63 *	1.38; -1.72	1.29; -1.81
Church Bay – Carmel/Cemlyn †	94.36	22.00	-4.32; -6.66 *	1.32; -1.02	1.12; -1.22
Church Bay – Bull Bay †	43.67	22.27	-3.85; -6.99 *	1.74; -1.40	1.56; -1.58
Carmel/Cemlyn – Bull Bay	2.47	22.45	n.a	n.a	n.a

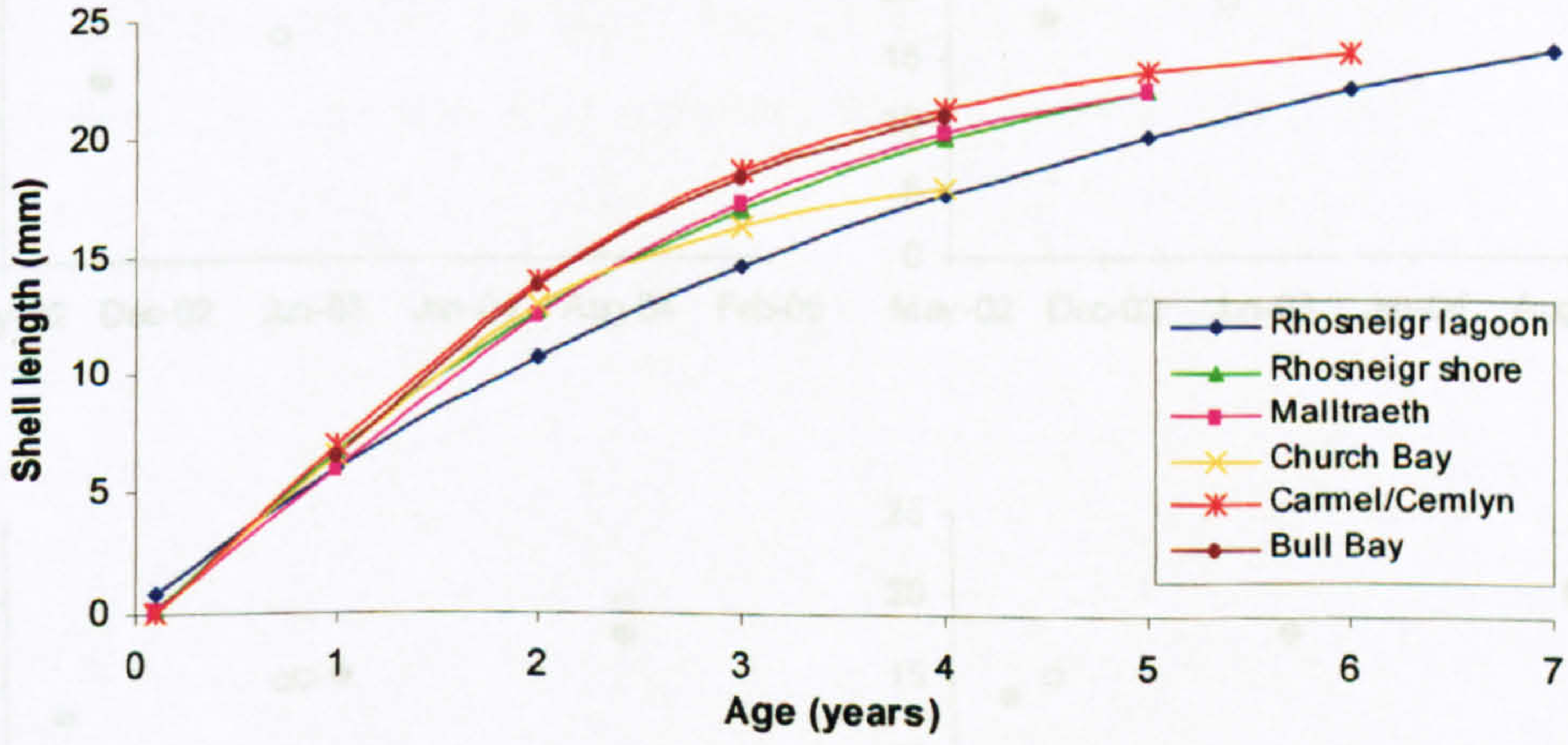
Figure 4.12 Shell length of *Nassarius reticulatus* estimated from the diameter of settlement and annual rings from the 6 locations a) Rhosneigr lagoon, b) Rhosneigr beach, c) Malltraeth, d) Bull Bay, e) Church Bay and f) Carmel Head/Cemlyn Bay. Von Bertalanffy growth curves (red lines) are fitted to the data and the estimated asymptotic length (L∞) is noted on each graph (dashed blue line).



Comparison of the von Bertalanffy growth curves from the 6 locations in pairs using Hotelling's T^2 test indicated that in most cases there were significant differences between the growth constants. The asymptotic maximum shell length (L∞) contributed most to the observed differences. The shell growth of whelks from the Rhosneigr lagoon, which is an isolated enclosed habitat subject to tidal emersion, was significantly different from all the other areas having the highest maximum asymptotic length (31.1

mm) and the lowest growth constant (0.21). As a result, the lagoon whelks grow slowly and attain a large size, which results in animals of older age; statoliths of these whelks contain up to 7 annual rings. By contrast, the growth of Church Bay whelks, which inhabit a wave-exposed shore located on the north-west coast of Anglesey, had the smallest L_∞ (19.7 mm) and the highest growth constant (0.69). As a consequence, these animals rapidly reach an overall small length, resulting in animals of younger age. The older whelks collected from Church Bay had up to 4 annual rings. No significant differences were found between the growth curves of *Nassarius reticulatus* from Bull Bay (L_∞=25.1 mm, K=0.52) and Carmel Head-Cemlyn Bay (L_∞=25.2 mm, K=0.54), which are both located along the relatively more sheltered north coast of Anglesey. Shell growth was similar between Malltraeth (L_∞=25.1 mm, K=0.48) and Bull Bay (L_∞=25.1 mm, K=0.52), and between Malltraeth and Carmel Head-Cemlyn Bay (L_∞=25.2 um, K=0.54). Although Malltraeth is located on the relatively exposed south-west coast of Anglesey the actual sampling site was close to "Malltraeth sands" a very sheltered estuary, thus perhaps explaining the similarities in shell growth rates between the north and south facing sites.

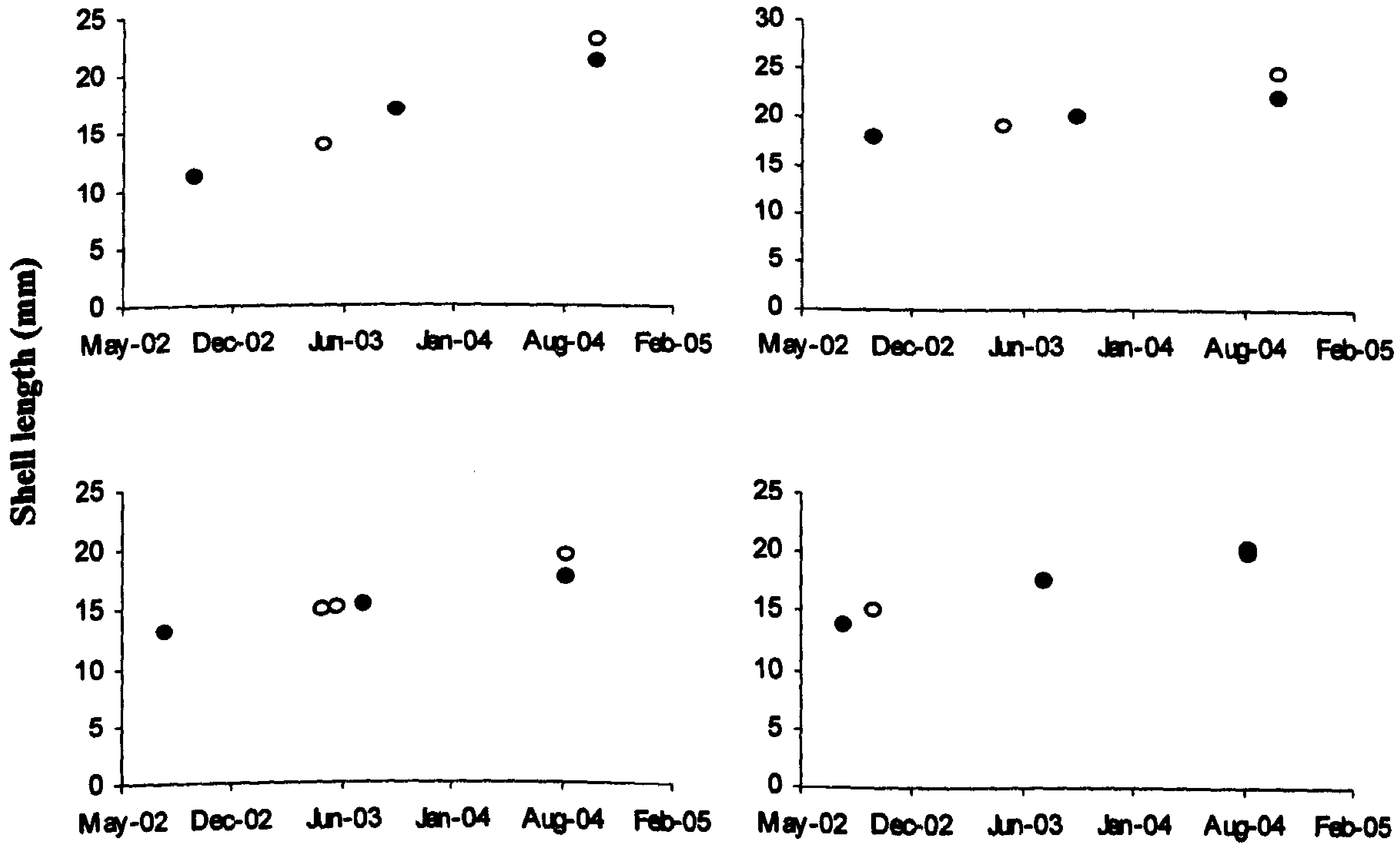
Figure 4.13 Summary of the von Bertalanffy growth curves of *Nassarius reticulatus* from six locations fitted to the shell lengths estimated from the statolith rings.



4.3.10 Comparison of shell length of *Nassarius reticulatus* estimated from statolith rings and from direct measurements of marked-recaptured whelks

The shell length of the marked *Nassarius reticulatus* was measured before they were released into the Rhosneigr lagoon, and then re-measured again every time they were recaptured, thus the shell length of these whelks was known for specific dates. Using the statoliths from 21 marked and recaptured individuals the shell lengths at the formation of the annual statolith rings were estimated from equation 4 (section 4.3.8), and compared with the actual shell lengths measured during recapture in order to estimate the reliability of the shell length measurements estimated from the statoliths. The data were normal (A^2= 0.274, p=0.656) and had equal variances (Bartlett's T=0.557, p=0.059). A 2-sample t-test indicated that there were no significant differences between the shell length estimated from the statolith rings and the shell length measured during recaptures (T= -0.82, p=0.414). Therefore an estimate of shell length using statolith diameter at the formation of annual rings from equation (4) allows a very good estimation of the shell length of gastropods during their lifetime. A comparison of the estimated shell lengths from the statolith rings with the measured shell lengths of 4 individuals are presented as representative examples in figure 4.14.

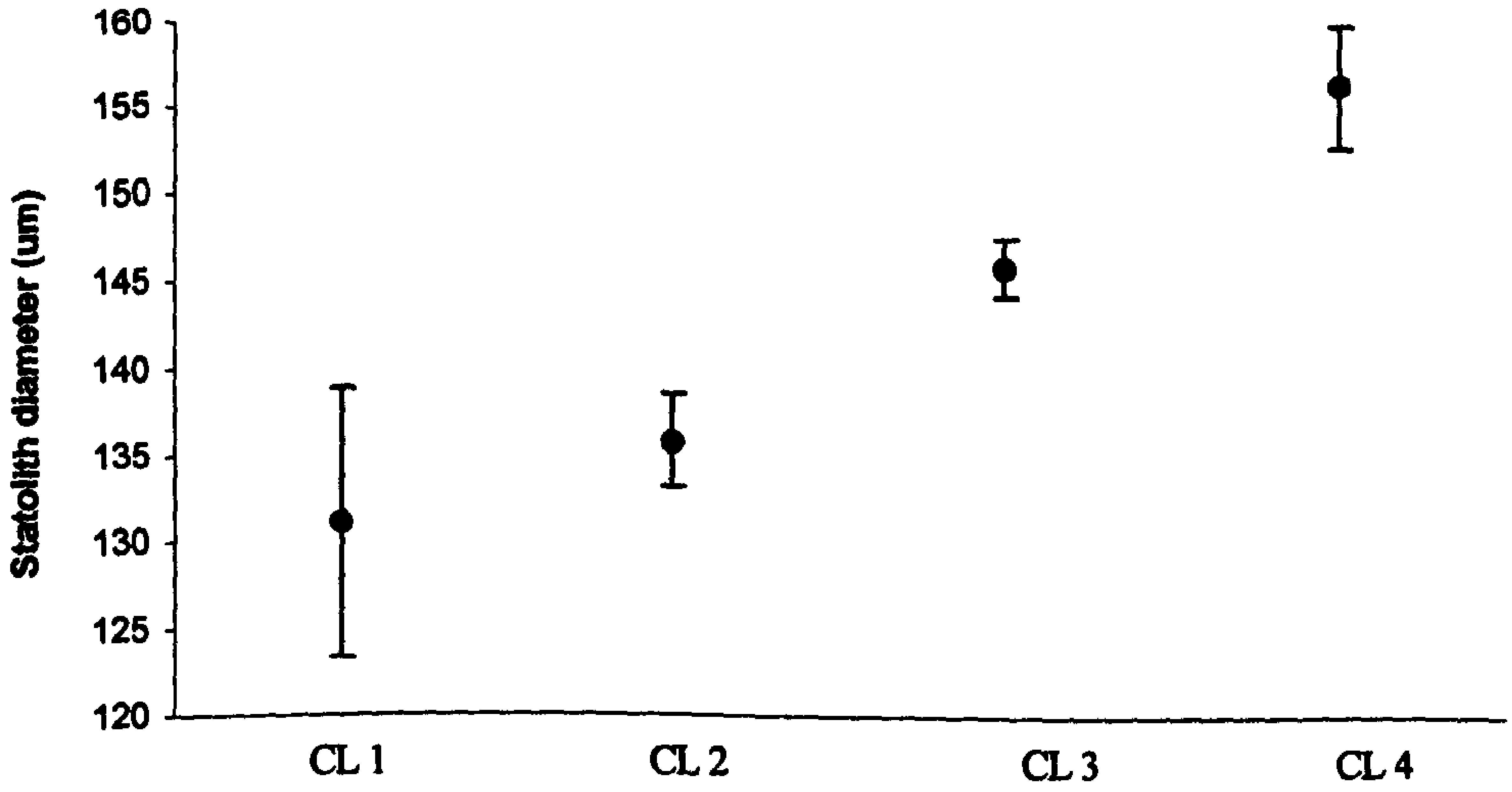
Figure 4.14 Comparison of the shell length (mm) of 4 marked and recaptured *Nassarius reticulatus* estimated from statolith growth rings (closed symbols) and from direct measurements of length during recaptures (open symbols) from the Rhosneigr lagoon.



4.3.11 Correlation of statolith diameter with shell morphology

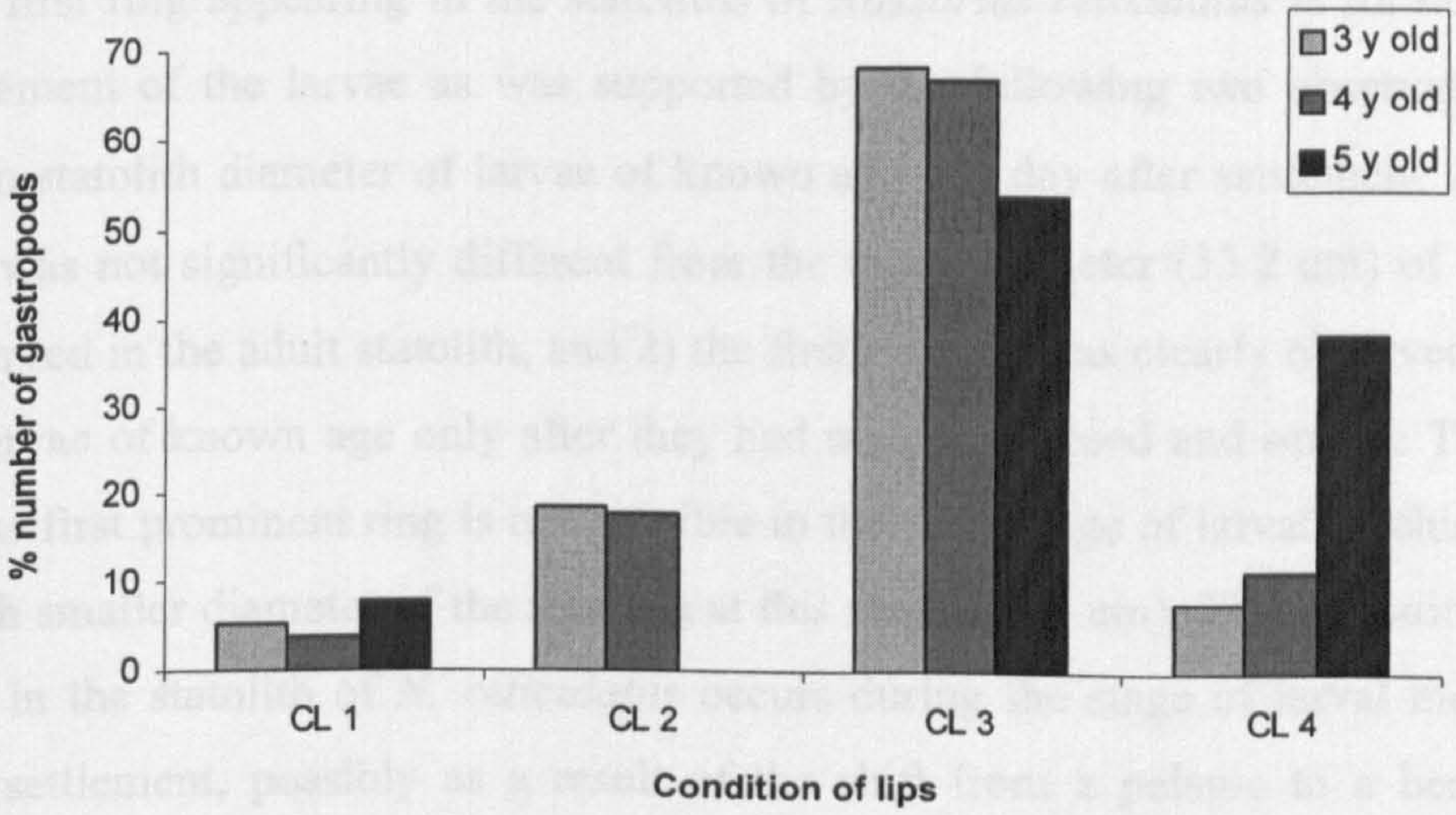
The thickness of the shell margin (lips) and the formation of teeth-like sculptures on the inner lip have been directly linked to mature age in gastropods (Fretter & Graham, 1984; 1994; Hughes, 1986) and have been extensively used as age indicators in several studies (Wada *et al.*, 1983; Appeldoorn, 1988). During the present study (see chapter 2, section 2.3.8) it was observed that larger *Nassarius reticulatus* had thicker shell lips with teeth formed on their inner lip (initially a row of single teeth that later develops into elongated teeth). Statolith diameters were compared between animals displaying the 4 categories of lip condition (see chapter 2, section 2.2.6). The data were normally distributed (CL1: $A^2= 0.454$, p=0.194; CL2: $A^2= 0.531$, p=0.160; CL3: $A^2= 0.247$, p=0.748; CL4: $A^2= 0.259$, p=0.677) and had equal variances (Bartlett's T= 2.842, p=0.417). A one-way ANOVA comparison indicated significant differences (F= 30.94, $p<0.001$) between the statolith diameter of the 4 shell lip stages. Animals with thin shell lips (CL1) had the smallest mean statolith diameter of 131.1 um (±3.89), whilst animals with thick shell lips and elongated teeth on the inner lip (CL4) had the biggest mean statolith diameter of 156.41 um (±1.78). Animals with thickened shell lips (CL2) had a mean statolith diameter of 135.81 um (±1.32) and animals with thick shell lips and single teeth (CL3) had a mean statolith diameter of 145.70 um (±0.83) (figure 4.15).

Figure 4.15 Mean statolith diameters (um) of *Nassarius reticulatus* with different shell lip condition (CL). The error bars represent the 95% confidence intervals of the means (1.96·SE).



Nassarius reticulatus were separated into age classes according to the number of rings in their statoliths and the percentage of whelks characterised by the 4 lip conditions (CL1-CL4) in each age class was calculated (see figure 4.16). In the 3-year old class 68.4% of the whelks displayed a CL 3 stage and 7.9% a CL 4 stage, whereas in the 4-year old class 67% of the whelks had CL 3 lips and 11.3 % had CL 4 lips. In the 5-year old class the percentage of animals with CL 4 lips increased to 38.5%, and 53.9% had CL 3 lips. Thus, it can be concluded that the thickening of shell lips and the formation of teeth on the inner lip are indeed characters which can be associated with older age classes of *N. reticulatus*. However, a high percentage of whelks from all the age classes displayed a CL 3 lip stage, and therefore thickness of the shell lips can only be used as an indicator of mature age and not as a method for determining empirically the age of *N. reticulatus*.

Figure 4.16 Percentage of *Nassarius reticulatus* of three different age classes (3-5 years old) with lip condition CL 1 to CL 4.



4.4 DISCUSSION

The statoliths of *Nassarius reticulatus* have a 3-dimensional elliptical shape. The pair of statoliths and their position in the foot tissue are visible through the thin larval shells in newly hatched larvae and for up to 5 weeks after hatching. Kingsley-Smith *et al.* (2005) were also able to observe the larval statolith of *Polinices pulchellus* through the shell of newly hatched larvae up to the age of 14 days. Each of the two semi-transparent statoliths is located inside separate statocysts. The ring patterns and diameters are similar between the pair of statoliths, thus either of the two can equally be used for analysis. Saurel (2002) reported there was no significant difference in the *Nucella* *lapillus* statolith surface area between the two statoliths of a pair. Microscopical examination of the *Nassarius reticulatus* statoliths revealed a clear pattern of 1 to 7 concentric rings around the nucleus. The nucleus of the statolith has a mean diameter of 5 um and is formed during the early embryonic stages. Saurel (2002) observed a nucleus with a diameter of only a few um in the embryonic statoliths of *Nucella lapillus*. Statolith diameter in developing embryos of *N. reticulatus* enclosed in egg capsules was 12.5 um, and by the time the larvae hatched the diameter had reached a size of 17.5 um. The statolith diameters of embryos and newly hatched larvae were exactly identical in all the individuals examined. Barroso *et al.* (2005b) indicated that the statolith diameter of *N. reticulatus* larvae at hatching was 16 um, and 6 days later it had reached a size of 20 um (non-fed larvae). In the present study the diameter of the first prominent ring (33.2 um) was validated as the settlement ring, and was similar in *N. reticulatus* collected from various sites around Anglesey. The size of statoliths after settlement was, however, significantly different between whelks from the different locations.

The first ring appearing in the statoliths of *Nassarius reticulatus* is formed during the settlement of the larvae as was supported by the following two observations: 1) The mean statolith diameter of larvae of known age one day after settlement was 34.0 um, and was not significantly different from the mean diameter (33.2 um) of the first ring observed in the adult statolith, and 2) the first ring (S) was clearly observed in statoliths of larvae of known age only after they had metamorphosed and settled. The formation of the first prominent ring is not possible in the early stage of larval hatching due to the much smaller diameter of the statolith at this stage (17.5 um). The formation of the first ring in the statolith of *N. reticulatus* occurs during the stage of larval metamorphosis and settlement, possibly as a result of the shift from a pelagic to a benthic habitat. Similarly the statolith microstructure of the squid *Psychroteuthis glacialis* consisted of two distinct zones (an inner opaque and an outer translucent zone) that reflected a habitat shift from the pelagic to the demersal phase of growth (Jackson, 1993).

Previous studies have suggested that the first prominent ring appearing in the statoliths of *Buccinum undatum* and *Nucella lapillus* is formed during the hatching of juveniles from their egg capsules (direct development) (Karageorgopoulos, 1997; Saurel, 2002). When Saurel (2002) compared the position of the first ring in adult *N. lapillus* with its position in the larval statolith at hatching no significant differences were found. Zacherl *et al.* (2003) indicated that the statolith diameter of *Concholepas concholepas* larvae at hatching was similar to the diameter of the first prominent ring which appeared in juvenile statoliths, indicating that the ring was formed during the transition from egg capsules to planktonic larval life (planktonic development). However, Richardson *et al.* (2005a) and Barroso *et al.* (2005b) assumed that formation of the first ring in statoliths of *Polinices pulchellus* and *Nassarius reticulatus* (planktonic development) occurred during settlement, and not during the larval hatching. In both species the diameter of the statolith during planktonic life (pre-settlement) was smaller than the diameter of the first ring in the adult statolith though lack of data on the statolith diameter of newly settled larvae could not validate this argument further. In the present study the evidence is unequivocal that the first ring is formed during settlement of the *N. reticulatus* larvae and not during hatching from the egg capsules. No rings were observed on larval statoliths before their settlement and the diameter of the larval statolith at hatching was far smaller than the diameter of the first prominent ring in adult statoliths. It is clear that formation of the first prominent ring in the statoliths is correlated either with larval hatching or settlement and differences have been observed between species examined until now. The formation of the first ring could possibly be correlated with the hatching phase in gastropods that are characterised by direct development (e.g. *B. undatum*, *N. lapillus*), whereas in species that spend a period of time in the plankton before they metamorphose (e.g. *P. pulchellus, N. reticulatus)* the formation of the first ring is correlated with the settlement phase.

In order to investigate when the first ring, following the settlement ring, was deposited the statolith diameters of post-juveniles of known age were compared with the mean diameter of the first ring in the adult statolith. The statolith diameter of 270-day old juveniles was 70 um and was significantly smaller than the mean diameter of the first ring in adults (82.7 um). However, the statolith diameter of 500-day old juveniles was 93.6 um and was not significantly different from the first ring diameter in adults. Thus it can be concluded that the first ring following the settlement ring is formed after the first year of life. The first ring can be clearly seen in photographs of *Nassarius* post-juveniles older than 1 year old. Hitherto no straightforward experimental evidence for the annual periodicity of statolith rings had been provided. Karageorgopoulos (1997) assumed that subsequent rings after the hatching ring in the statoliths of *Buccinum undatum* were formed annually, as a result of cessation of growth during winter, but there was no experimental evidence to support this. According to Richardson *et al.* (2005a) and Barroso *et al.* (2005b) the formation of rings in *Polinices pulchellus* and *Nassarius* *reticulatus* statoliths is probably annual. The estimated shell lengths of *P. pulchellus* at the formation of the first and second prominent statolith rings were similar with the shell lengths of 1 and 2-year old animals that were described from the length frequency analysis of the population (Richardson *et al.*, 2005a). Similarly, length-at-age data from statolith rings of *N. reticulatus* were in agreement with shell length at age estimated from length frequency analysis and from the rings on the outer surface of the shell (Barroso *et al.*, 2005b). Richardson *et al.* (2005b) suggested that the rings in the statoliths of *Neptunea antiqua* were deposited annually, since the number of statolith rings was in agreement with the number of seasonal Mg:Ca ratio cycles in the shells. During the present study the statolith analysis of individuals of known age reared in the laboratory validated the conclusion of an annual ring periodicity in *N. reticulatus* statoliths. Further studies using chemical markers in the statoliths of individuals of known age reared in the laboratory could reveal more information about the periodicity of the growth rings in the statoliths of other gastropod species. The periodicity of statolith rings has been validated for several squid species using chemical markers, such as tetracycline (Dawe *et al.*, 1985; Lipinski, 1986; Jackson, 1989; 1990a; 1990b; Lipinski *et al.*, 1998; Dimmlich & Hoedt, 1998), strontium (Dawe *et al.*, 1985; Hurley *et al.*, 1985) or calcein (Jackson, 1990a).

Larval statoliths of other gastropod species examined in previous studies revealed a series of weaker lines that could be interpreted as daily rings. Daily rings have been demonstrated in the larval statoliths of *Littorina scabra* (Bell, 1983; Bell, 1984) and in the larval statoliths of the queen conchs *Strombus costatus* and *S. gigas* (Grana-Raffucci & Appeldoorn, 1997). When the statoliths of *Concholepas concholepas* larvae were examined under a microscope a series of concentric rings were observed from the core to the edge of the statolith (Zacherl *et al.*, 2003). These rings were observed both in juveniles and non-hatched larvae and they could possibly be daily increments, although Zacherl *et al.* (2003) did not present any experimental observations to support their conclusions. Similar weaker rings were also observed in the statoliths of *Nucella lapillus, Polinices pulchellus and Neptunea antiqua* adults, but there was no evidence to suggest that they have a daily periodicity (Saurel, 2002; Richardson *et al.*, 2005a; 2005b). No such patterns of weak background rings that could be formed with a daily or even a monthly periodicity were observed in the statoliths of *Nassarius reticulatus* (Barroso *et al.*, 2005b; present study). Richardson *et al.* (2005a) suggested that since such weaker rings appeared in the statoliths of predatory species (*N. lapillus*, *P.*

pulchellus, *N. antiqua*, *C. concholepas*) and not in the statoliths of scavengers (*N. reticulatus*) their formation could be correlated with the feeding lifestyle of these gastropods. The energetic cost of drilling prey shells, sometimes up to several days, may direct energy from statolith deposition, thus producing weaker rings (Richardson *et al.*, 2005a). Factors which might influence the formation and periodicity of weaker statolith rings could be correlated with feeding frequency, drilling activity and environmental disturbances (Richardson *et al.*, 2005a). Deposition of less prominent statolith rings has yet to be examined in the statoliths of species where such weaker lines appear, in order to establish their periodicity and validate their use for studying ecological patterns of larval dispersion and life-strategies.

The statoliths from gastropod species studied so far have demonstrated significant differences in the degree of definition of the growth rings and in the clarity of the ring patterns. Poor definition and high variability in the diameter of the growth rings prevented an adequate description of statolith micro-ring formation in *Nucella lapillus* (Saurel, 2002). Differences in the biochemical composition of the statolith and the statocyst endolymph can result in different crystallisation processes and thus different statolith microstructure between species. For example Bettencourt & Guerra (2000) noted that growth increments were better defined in the statoliths of *Loligo vulgaris* than in the statoliths of *Sepia officinalis*. The higher proportion of organic matter in the statoliths of *L. vulgaris* resulted in the formation of better defined growth increments (Bettencourt & Guerra, 2000). In addition, the statocyst endolymph in *L. vulgaris* has a higher protein content that smoothers the aragonite crystal edges, thus allowing a uniform and continuous deposition of organic matter and thereby a better visualisation of the growth increments (Bettencourt & Guerra, 2000). It has also been noted that the growth rings at the circumference of the statoliths in older (larger) *Buccinum undatum* and *Nucella lapillus* were not easily distinguished (Karageorgopoulos, 1997; Saurel, 2002). As the animals grow older, growth slows down resulting in the formation of less wide increments at the statolith edge, where growth rings frequently cannot be easily identified. In addition, due to the reduction in the proportion of organic matter in statoliths with increasing age, the statoliths become more calcified and increment definition is reduced (Radtke, 1983; Bettencourt & Guerra, 2000).

Having established the annual periodicity of the statolith growth rings in *Nassarius reticulatus* the seasonal pattern of the growth increments formation was investigated. A dark thin growth ring formed in the statolith alternates with a wider light ring and both together are defined as a "growth increment". The distance between the last dark growth ring and the circumference of the statolith represents the part of a new increment formed in any particular time. Marginal increment analysis has been used for validating the timing and periodicity of increments and utilises apparent temporal trends in the formation of increments on the growing edge of fish otoliths (Ewing *et al.*, 2003). The formation of growth increments in the statoliths of *N. reticulatus* ceased during winter when the seawater temperature was low. When temperatures increased again in spring, incremental growth of the statoliths accelerated until the early summer. The timing of the formation of a statolith increment was coincident with the annual increase in seawater temperature from 6.9 °C in March to 17.9 °C in August. However, during mid and late summer, when energy is probably allocated to reproduction, growth slowed down and the rate of increment formation in the statoliths was also reduced. Formation of the light wider ring occurred between spring and early summer when most of the assimilated energy was allocated into somatic growth. Subsequently, formation of the dark ring occurred between late summer and winter, when statolith growth slowed down, probably due to reproduction initially and in response to cooler seawater temperatures later.

Radtke *et al.* (1985) suggested that the wider hyaline areas (light wide rings) in squid statoliths corresponded to periods of fast growth which occurred in the summer, while the thinner opaque areas (dark rings) were related to periods of slow growth during the winter. Similarly, the prominent dark rings in otoliths of pilchard are formed when seawater temperatures are colder than the annual average (Thomas, 1983). However, in otoliths of *Notolabrus fucicola* the annual rings (dark zones) mark the transition from slow winter growth to fast spring and summer growth, rather than coinciding with periods of slow growth (Ewing *et al*, 2003). According to Hostetter & Munroe (1993) the formation of annual marks on opercle bones of tautog *Tautoga onitis* was concurrent with the spawning season. In future studies collection of statolith seasonal samples in more closely spaced timings, e.g. fortnightly or monthly periods, could provide more detailed information on the exact process of ring formation in the statoliths. For example in statoliths of *Nassarius reticulatus* collected between April and May the width of the light ring would be expected to be very small confirming that the light ring had only begun forming during the spring period.

An exponential relationship (equation 4, section 4.3.8) was established between shell length and statolith diameter of *Nassarius reticulatus* in order to reconstruct the life history and growth patterns of gastropods from the larval stages up to the maximum size reached in adults. The equation established included measurements of larvae of all ages, juveniles and adults and can be used to estimate the shell length of *N. reticulatus* within the range 0.3 to 30 mm at formation of the annual statolith rings. The same equation can be utilised to estimate the shell length of male, female or imposex affected *N. reticulatus*, collected during different seasons and from different locations around Anglesey. Saurel (2002) indicated that the relationship between shell length and statolith diameter in *Nucella lapillus* was also not significantly different between the different shores around North Wales. Radtke *et al.* (1985) noted that the relationship between body length of oyster toadfish *Opsanus tau* and the diameter of otoliths was similar for both sexes. Lipinski (1980) also did not find any differences in the relationship of statolith size and body size in the squid *Illex illecebrosus* between different areas, sexes or years. Barroso *et al.* (2005b) indicated that the relationship between shell length and statolith diameter of *N. reticulatus* was significantly different between three sampling sites in the Ria de Aveiro (Portugal), however, a very dissimilar size range was used from each of the stations examined, which may have contributed to those differences.

The relationship between length of an organism and statolith diameter has been used in previous studies as a tool for the back-calculation of size and reconstruction of growth patterns in several species of cephalopods, such as *Alloteuthis subulata* (Lipinski, 1986), *Sepia hierredda* (Raya *et al.*, 1994), and in the gastropods *Polinices pulchellus* (Richardson *et al.*, 2005a) and *Nassarius reticulatus* (Barroso *et al.*, 2005b). The back-calculation of age-specific growth rates from increment width measurements has been widely recognised as a powerful application to the analysis of otolith microstructure (Re & Goncalves, 1993). Back-calculation of shell length at the formation of each annual ring can be performed by measuring the diameter of the statolith ring and substituting this value in an equation relating statolith size to length. This method provides important information about growth patterns and life strategies of organisms during their lifetime in different ecosystems. Thus, for example, variations in shell size at settlement between different areas can reveal differences in the length of time the gastropods spend in the plankton, and settlement rings can be used to determine the time of the year when each individual was released. According to Richardson *et al.* (2005a) larger settlement rings could be the result of earlier recruitment of individuals during the spring, thus their planktonic development at low seawater temperatures would last longer.

The equation established during the present study was used to estimate shell length of *Nassarius reticulatus* from statolith diameter at the formation of the settlement and annual rings. The length-at-age data produced were used to construct von Bertalanffy growth curves and to describe the growth pattern of *N. reticulatus*. Comparisons of the VBG curves provided information about variations in growth of *N. reticulatus* of different gender. No significant differences were observed between the growth rates of female and male *N. reticulatus* or between females and imposex affected females during the present study. Similarly, the growth rates of male and female *N. reticulatus* reared under laboratory conditions were similar (see chapter 3). Curtis (1995) has shown that male and female *Ilyanassa obsoleta* have similar growth rates. Similar growth rates between males and females have been reported for other gastropod species, such as *Polinices duplicatus* (Edwards & Huebner, 1977) and *Concholepas concholepas* (Rabi & Maravi, 1991). However, gender induced differences in growth rates have been observed in the gastropods *Shaskyus festivus, Ocenebra poulsoni, Japeuthria ferrea* and *J. cingulata* where the females had a faster growth rate than the males (Fotheringham, 1971; Ota & Tokeshi, 2000). Such variations in growth between males and females may reflect differences in the utilisation of energy reserves during reproduction, whilst differences in growth between normal females and imposex affected females might be indicative of abnormalities of growth due to TBT pollution. Although in the present study the imposex affected female *N. reticulatus* had similar growth to normal females, several studies have reported that TBT pollution affects growth rates. For example TBT pollution caused abnormal shell growth in oysters (Dyrynda, 1992), and reduced growth rates in *Scrobicularia plana* larvae (Ruiz *et al.*, 1995). Imposex affected females of *Hexaplex trunculus* and *Stramonita haemastoma* had larger shells than males, because the sterile females allocated more energy to shell growth rather than to reproduction (Rilov *et al.*, 2000).

The shell length of *Nassarius reticulatus* at the formation of the settlement and annual rings was estimated for whelks that were collected from 6 different locations in Anglesey. No significant difference in the mean shell length of *N. reticulatus* larvae at settlement was observed around Anglesey, indicating that the larvae spend a similar period of time in the plankton before settling onto the sediment. The mean shell length at settlement estimated from adult statoliths (0.8 mm) was similar to the mean shell length measured in recently settled larvae (0.78 mm). Lebour (1937) and Fretter & Graham (1984) also reported that the shell length of *N. reticulatus* larvae at settlement was 0.7-0.8 mm. Shell length during the formation of the first, second, third and fourth annual rings was, however, significantly different between the 6 locations. It is evident that although all whelks had the same size at settlement, the size reached at a particular age is variable between different localities. These differences have probably arisen due to different environmental factors such as wave exposure, seawater temperature range, food availability, or even genetic factors that promote adaptation to different habitats.

Comparison of the VBG curves produced from the length-at-age data revealed significant differences in the growth pattern of whelks between the 6 sampling areas. The asymptotic maximum shell length (L_∞) contributed the most to these differences. Adults collected in the Rhosneigr lagoon had the highest maximum asymptotic length (31.1 mm) and the lowest growth constant (0.21). Growth patterns in the lagoon revealed a slower growth rate resulting in larger sized whelks over a greater period of time, thus animals collected from this locality were older than animals collected from the other areas. Up to 7 annual rings were observed in the statoliths of whelks collected from Rhosneigr lagoon, while fewer rings were observed in the statoliths of whelks from the other locations. The growth of the Church Bay whelks, located on the north-west coast of Anglesey had the lowest maximum asymptotic length (19.7 mm) and the highest growth constant (0.69). Rhosneigr lagoon and Church Bay are two very dissimilar sites with the lagoon being classified as very sheltered (6-7) on the Ballantine scale, whilst Church Bay is classified as exposed (3). No significant differences were found between the growth curves of *Nassarius reticulatus* collected from Bull Bay and the Carmel Head-Cemlyn Bay areas; both sites are located on the north coast of Anglesey and are classified as sheltered (6) on the Ballantine exposure scale. Moreover, growth patterns in whelks from these two north Anglesey sites were similar to the growth of Malltraeth whelks. Malltraeth is located on the semi-exposed (4) south coast of Anglesey. However, the actual sampling site was close to "Malltraeth sands" a sheltered estuary, thus perhaps explaining the similarity in the growth between whelks from north and south located areas. Growth patterns at Rhosneigr beach were not similar to any other area, although this site is also classified as "sheltered" (6) on the Ballantine scale and is located close to Malltraeth on the south-west coast of Anglesey.

However, Rhosneigr is characterised by a higher biological variability (see Brazier *et al.*, 1999) than the northern areas of Bull Bay and Carmel-Cemlyn and the sandy Malltraeth site.

Comparison of the VBG curves, which were constructed from length-at-age data using statolith increments in the present chapter, indicated that *Nassarius reticulatus* from the sheltered site Rhosneigr lagoon had the lowest growth constant and the highest asymptotic length compared with whelks from the most exposed site Church Bay. Similarly, in chapter 3 the MPA analysis of the length frequency distributions indicated that whelks from the intertidal lagoon in Rhosneigr had the lowest K and the highest L_∞. Differences in shore exposure and predation pressure amongst the different sites may account for differences in the observed shell sizes and growth rates. In an enclosed lagoon ecosystem, which is characterised by lack of wave exposure and, as a result, greater populations of predatory crabs, thickening of the shell is promoted instead of shell growth in terms of length (Wellington & Kuris, 1983). *N. reticulatus* collected from the Rhosneigr lagoon had a slower growth rate (measured as shell length) presumably because more energy was invested in thickening of the shell to protect the whelk against predation. The morphometrical analysis in chapter 2 confirmed that *N. reticulatus* from the sheltered sites had longer and thicker shells compared with conspecifics from the exposed sites. Production of a thick shell is energetically costly (Palmer, 1992) and may restrict the maximum rate of body growth (Palmer, 1981). Since there is a maximum rate at which calcification can occur, when more material is devoted to shell thickening less is available for linear shell growth (Trussell & Smith, 2000). Slow growing adults of *Bembicium vittatum* displayed thicker shells than fast growing adults (Parsons, 1997a). In the presence of water tainted with the chemical signature of potential predatory crustaceans, the shells of *Nucella lapillus* and *Littorina obtusata* grow more slowly and become thicker (Palmer, 1990; Trussell, 1996; Trussell & Smith, 2000). Thick-shelled individuals of *Littorina* sp. were less preferred by shore crabs and withstood significantly more force than thin-shelled individuals (Boulding & Van Alstyne, 1993). Although *N. reticulatus* in sheltered areas had a lower growth rate (K), the maximum asymptotic length of these individuals was larger (L_∞) resulting in animals of larger size. The formation of larger shells in sheltered environments offers effective protection against predation (Crothers, 1973; Parsons, 1997a). The relative shell strength increases roughly with the 1/3 power of shell length, thus gastropod shells of greater length are stronger than the ones of smaller length (Tokeshi *et al.*, 2000).

Juanes (1992) indicated that crab and lobster predators prefer small-sized gastropod shells when they are offered a range of sizes. Large shells require more energy to break them, which increases the probability of damage to the predators claws, whereas small shells are weaker and require exponentially less force to achieve breakage (Juanes, 1992).

As in the present study, differences in statolith and otolith microstructure have also been noted among squids originating from different geographic areas. Juveniles of *Illex argentinus* hatched in the cold waters of the Argentine basin had well defined narrow daily increments (2-3 um), while juveniles hatched in the warm waters of the Patagonian shelf displayed a faster growth rate reflected in wider (4-5 um) and less defined increments (Arkhipkin, 1993). Direct application of size at age information from otoliths has been successfully used to identify growth variations in fish larvae from different geographical areas (Brown & Bailey, 1992; Re & Goncalves, 1993). Moksness (1992) was also able to use variations in the widths of otolith increments to identify North Sea herring from different geographical areas.

The thickness of the shell margin (lips) and formation of teeth-like sculptures on the inner lip have been directly linked to maturity in gastropods and have been extensively used as age indicators for *Strombus gigas* (Appeldoorn, 1988), *S. luhuanus* (Wada *et al.*, 1983), and *Nucella lapillus* (Hughes, 1986). According to Fretter & Graham (1994) as long as a gastropod is immature and further shell growth is likely to occur, the outer lip remains thin and sharpened. When maturity is reached spiral growth ceases and the only further deposition of calcareous material leads to the formation of a thicker lip at the aperture of the shell and to the appearance of teeth projecting into the opercular aperture (Fretter & Graham, 1994). After maturity the inner lip of the shell of *Nassarius reticulatus* thickens and forms a set of ridges with 6-9 longitudinally elongated teeth (Fretter & Graham, 1984). Barroso *et al.* (2005a) suggested that thickening of shell lips in older *N. reticulatus* was associated with a series of thin micro-growth internal bands that accumulate at the edge of the lip. The micro-growth bands deposited on the lip of younger animals are wider resulting in a thinner shell edge (Barroso *et al.*, 2005a). In the present study *N. reticulatus* with thin shell lips had smaller statoliths and smaller shell lengths, whereas animals with thick shell lips and formed teeth had bigger statoliths and larger shells. The thickening of shell lips and the formation of teeth are morphological characters associated with mature age classes, however, due to the fact that mixed age classes were present in each of the shell lip condition categories, shell thickness and formation of teeth can only be used as rough indicators of older and more mature whelks. Shell length and shell lip thickness can be severely affected as a result of damage due to predation or erosion, while statoliths are completely protected and isolated from the external environment, thus information concerning the past history of the organisms can not be modified.

The existence of a positive relationship between body size and statolith size in cephalopods supports the view that the cephalopod statolith grows in concert with the rest of the animal and that increments are laid down regularly during growth (see Radtke, 1983; Rodhouse & Hatfield, 1990). However, in several cases relating to otolith analysis in fish or statolith analysis in cephalopods discordance between somatic growth and otolith/statolith growth has been observed. Jackson (1994), for example, found that slow growing *Loligo chinesis* during the winter had larger statoliths than similar sized faster growing summer conspecifics. Studies on fish otoliths have also documented that faster growing fish such as *Poecilia reticulata, Morone saxatilis* and *Theragra chalcogramma* have smaller otoliths than similar sized slow growing fish (Secor & Dean, 1989; Reznick *et al.*, 1989; Brown & Bailey, 1992). Such irregularities have been described as the Lee's phenomenon (Ricker, 1969) and can result in an under-estimation of size using statolith rings, compared to direct measurements of size from the population (Campana, 1990). During the present study careful examination of the data did not reveal that larger statoliths were produced by slower growing gastropods of similar size (e.g. from the Rhosneigr lagoon) compared with faster growing conspecifics (e.g. from Church Bay). The relationship established between statolith diameter and shell length and statolith diameter was not significantly different between areas with variable growth rates. Shell growth of *Nassarius reticulatus* is in full accordance with statolith growth, thus the description of the growth patterns and life history events can be revealed from statolith increments. Furthermore, validation of estimated shell length at a particular age from the statolith diameter at the respective annual rings, was established using marked and recaptured animals. The shell growth of marked individuals, released into the natural environment, was monitored throughout a period of two years and was compared with the shell length estimated from their statolith annual rings. No significant differences were found between the measured shell length of marked-recaptured whelks and the shell length estimated from statolith rings.

Statolith diameters at formation of the annual rings provided a reliable estimation of length-at-age data.

Three different methods were used in the present study to estimate *Nassarius reticulatus* length-at-age data: a) the graphical method MPA based on the Bhattacharya's analysis of the length frequency distributions (chapter 3), b) the computerised method LFDA using a maximum likelihood-based analysis of the length frequency distributions (chapter 3) and c) the estimation of shell length at formation of statolith annual rings (current chapter). Estimates of shell length of *N. reticulatus* at different ages using the 3 methods are summarised in table 4.10, together with data for other regions retrieved from the existing published literature. As indicated in chapter 3, growth measured in marked and recaptured whelks was more similar with growth estimated from the MPA analysis, than with growth estimated from the LFDA method. The LFDA analysis described only 4 age classes, whereas the MPA analysis described 5 age classes. However, the estimation of shell length based on statolith rings revealed more age classes (7) for the Rhosneigr lagoon population and a slower growth rate than the MPA analysis. According to the MPA analysis the 5-year old *N. reticulatus* in Rhosneigr lagoon were 27-28.9 mm, while the statolith analysis indicated that 7-year old whelks had reached a size of only 24.5 mm. The annual periodicity of statolith rings was validated using individuals of known age in the present chapter, and furthermore the estimation of length-at-age data using statolith rings was in full accordance with the growth increments measured in marked and recaptured whelks. Thus, it is suggested that the use of statoliths for the determination of age and growth of *N. reticulatus* is a more reliable method than the analysis of length frequency distributions which resulted in overestimated growth rates.

A comparison of the length-at-age data determined from the statolith rings of *Nassarius reticulatus* between the Anglesey population and populations from other regions (Sweden, Netherlands, Portugal) revealed some geographical variations (see table 4.10). *N. reticulatus* collected from Anglesey had similar shell length at age (statolith method) with individuals collected from Sweden (length frequency analysis) and from Portugal (statolith method and length frequency analysis), during their first 3 years of life. Growth of *N. reticulatus* in the Ria de Aveiro (Portugal) was greater for the 4 and 5-

Table 4.10 Estimates of shell lengths (mm) of *Nassarius reticulatus* during the first 5 years of life from Rhosneigr intertidal lagoon and subtidal beach using length frequency analysis methods (LFDA, MPA) and statolith rings. For comparison shell lengths of *N. reticulatus* from several localities indicated in the literature are also presented.

Shell length (mm)							**Locality**	**Method & Citation**
1st year	2nd year	3rd year	4th year	5th year	6th year	7th year		
5.9	10.9	14.3	17.6	20.6	22.2	24.5	Rhosneigr lagoon Anglesey, UK	Statoliths, chapter 4
7-8.9	19-20.9	25-26.9	27-28.9	-	-	-		LFDA, chapter 3
7-8.9	15-16.9	23-24.9	25-26.9	27-28.9	-	-		MPA, chapter 3
6.7	13.0	17.2	20.2	22.4	-	-	Rhosneigr beach Anglesey, UK	Statoliths, chapter 4
7-8.9	15-16.9	21-22.9	25-26.9	-	-	-		LFDA, chapter 3
7-8.9	15-16.9	21-22.9	23-24.9	25-26.9	-	-		MPA, chapter 3
5-6.7	10.9-13.6	14.3-19.1	17.6-21.4	20.6-22.4	-	-	Anglesey, UK (all areas)	Statoliths chapter 4
5.5	11	16.5	-	-	-	-	Gullmar fjord, Sweden	Length frequencies (Tallmark, 1980)
6.8	16	-	-	-	-	-	Grevelingen lake, Netherlands	Length frequencies (Lambeck, 1984)
6.7-7.8	12.1-14.5	18-19.5	22.7-23.6	25-27	-	-	Ria de Aveiro, Portugal	Length frequencies (Barroso *et al.*, 2005a)
4.6-5.3	12-13.5	18.5	-	-	-	-		Statoliths (Barroso *et al.*, 2005b)

year age classes (only length frequency analysis available) than growth of the Anglesey populations (statolith method). Individuals from Portugal reached a size of 25-27 mm in their fifth year, while whelks of the same age from Anglesey had only reached a size of 20.6-22.4 mm. However, if the results of the length frequency analyses of both populations are used in the comparison, the shell lengths of the older age classes (fourth and fifth year) of *N. reticulatus* estimated from the MPA analysis in Rhosneigr beach were similar with shell length estimates from length frequency analysis in Portugal. The higher seawater temperatures and the longer warm periods may be responsible for the increased growth of *N. reticulatus* in Portugal. Lambeck (1984) indicated that *N. reticulatus* in the recently colonised saline lake Grevelingen (Netherlands) had already reached a size of 16 mm in their second year, whereas whelks of the same age from Sweden, Portugal or Anglesey were much smaller. Lack of competition and increased food availability could result in the higher growth rates recorded in lake Grevelingen. Apart from the expected variability in growth rates due to the different environmental conditions between regions, the application of length frequency analysis for age determination may be responsible for the increased growth rates of *N. reticulatus* observed in the other regions. As was indicated above, growth of *N. reticulatus* in the intertidal lagoon was over-estimated when length frequency analysis was used, compared with estimation of shell length from statolith annual rings.

Jackson *et al.* (2000) indicated that size frequency analyses based on computer methods, which were actually constructed for fisheries studies, are not appropriate for estimating growth curves of squid populations and suggested that estimation of growth patterns with the use of daily growth lines on squid statoliths is a more realistic method. Similarly, several studies that have used analyses of size-frequency distributions indicated the unsuitability of those methods for the study of gastropod growth throughout the size range of the animal (Moore, 1937; 1938; Blackmore, 1969; Feare, 1970b). Erosion of the apical end of the shell in the field may mask small increases in shell length and lead to incorrect measurements of growth, especially in periods of low growth (Ekaratne & Crisp, 1984). Yamaguchi (1977) reported that the shell apex of the coral reef gastropod *Cerithium nodulosum* suffered progressive erosion even during the juvenile stages. The identification of the cohorts becomes impossible as the animals approach a larger size and the growth rates are reduced (Blackmore, 1969). Absence of growth over a 2-year period of study has been recorded for older individuals of *Nucella* *lapillus*, *Littorina littorea* and *Melaraphe (Littorina) neritoides* (Williams, 1964; Feare, 1970b; Hughes & Roberts, 1980). Overlapping of older age classes has been observed in length frequency distributions of *Nassarius obsoletus* (Scheltema, 1964), *N. reticulatus* (Barroso *et al.*, 2005a), *Littorina nigrolineata* (Hughes, 1980), *L. littorea* (Moore, 1937) and *Morula marginalba* (Moran *et al.*, 1984). Due to the size-dependent mortality and predation in most species, growth curves based on size-frequency distributions can lead to erroneous estimates of growth rate (Ekaratne & Crisp, 1984). Furthermore, in populations where recruitment is not consistent through space and time, size frequency distribution analysis should not be used in growth studies because there are no clear year groups present from which growth could be determined (Moore, 1938; Yamaguchi, 1977; Ekaratne & Crisp, 1984).

The decreased resolution of shell length measurements in older animals indicated that length frequency distribution analysis may not be suitable for long-lived small-sized species, because it can only detect gross changes in growth rates (Ekaratne & Crisp, 1984). Pronounced individual plasticity in growth and protracted spawning, which result in size classes overlapping and multiple cohorts, could lead to confusing length frequency modes and difficulties in the interpretation of the length frequency analysis (Jackson *et al.*, 2000). Hilborn & Walters (1992) also indicated that attempts to use length frequency analysis for species that do not exhibit unambiguous modes is misguided. According to Grant *et al.* (1987) the presence of detectable growth records (e.g. growth rings) on the hard parts of invertebrates can give straightforward information about the growth rate and age composition of a population, although the periodicity of the formation of growth records has to be validated. Ekaratne & Crisp (1984) indicated that growth estimations based on shell micro-growth band measurements in intertidal gastropods had higher resolution, was far more sensitive than size-frequency distribution analysis, and was able to detect growth even in animals of larger classes. Gray (1997) also indicated that the growth rates of the bivalve *Mytilus edulis chilensis* determined using the method of Bhattacharya (MPA) were considerably higher than those estimated from annual growth checks on the shells. The length frequency distributions had limited application for the determination of the age of mussels due to the overlapping of older cohorts, and only the first 2-3 years age classes following recruitment could be identified (Gray, 1997). Analytical methods for length frequency distributions may only be used for short-lived rapidly growing species where no overlapping of annual cohorts occurs. For example, the LFDA analysis described successfully the growth pattern of *Polinices pulchellus* (2 years longevity) and was in accordance with the estimated length-at-age data from statolith rings (Richardson *et al.*, 2005a). Also the estimated shell lengths of *Nassarius reticulatus* in the Ria de Aveiro from statolith rings, were in good agreement with the length frequency analysis (see table 4.10), however only young individuals with up to 3 annual rings were examined (Barroso *et al.*, 2005b).

During the present study the formation of the first prominent ring during the settlement of the larvae, and the annual periodicity of the following prominent rings in the statolith of *Nassarius reticulatus* has been established using animals of known age reared in the laboratory. The light wider rings in the *N. reticulatus* statoliths are formed during spring and early summer when more energy is available for somatic growth, whereas the dark thinner rings are formed between late summer and winter when growth is suppressed due to reproduction initially and low temperatures later. Determination of the age of gastropods by counting the growth rings in the statoliths is now possible, and is based on the fact that each dark ring represents one year of age. Additionally, an equation relating shell length and statolith diameter has been described, and has been used for estimating the shell length of *N. reticulatus* during the formation of each annual ring. The same equation can be applied to larvae, juvenile and adult *N. reticulatus* (size range 0.3-30 mm), males, females and imposex affected whelks, that are collected in any season of the year from different locations around Anglesey. The estimation of length-at-age data from statolith increments has been validated using marked individuals for monitoring of growth. Shell and statolith size were similar for whelks collected from different localities up to the settlement stage. However, significant differences in statolith and shell size at formation of the annual rings, and also significant variations in growth patterns, were observed between *N. reticulatus* from different areas. Growth patterns of male, female and imposex affected *N. reticulatus* were similar. The condition of shell lips, described as thickness of shell lips and formation of teeth on the inner lip, is indeed correlated with whelks of older age though it is not a reliable method for determination of age in *N. reticulatus*. Estimation of shell length from statolith rings is suggested as a more appropriate method for determination of length-at-age in *N. reticulatus* than the length frequency distribution methods, which can overestimate growth of whelks due to overlapping of older age classes in the population.

CHAPTER 5

Egg capsule production by *Nassarius reticulatus*

5.1 INTRODUCTION

The sexes are separate in *Nassarius reticulatus* and reproduction involves copulation and internal fertilisation (Hayward & Ryland, 2000). The reproductive cycle of *N. reticulatus* is synchronised in males and females (Barroso & Moreira, 1998) and the whelks become sexually mature at the age of ~ 4 years (Tallmark, 1980; Bryan *et al.*, 1993). Barroso *et al.* (2005a) indicated that female *N. reticulatus* mature when they are 4 to 5 years old, about one year later than males. Breeding of *N. reticulatus* throughout Europe takes place mostly in inshore waters during spring and summer, usually from March to August, and more rarely during winter and autumn (Lebour, 1931). Variations have been found between populations from different areas. Fretter & Graham (1984) reported that breeding of *N. reticulatus* occurs from March to August in Plymouth (UK), from late April to late August in the Eastern Channel (UK) and from January to May near Kiel (Germany). In Isefjord (Denmark) spawning of *N. reticulatus* was observed between April and August (Rasmussen, 1973). Breeding of *N. reticulatus* in the Firth of Clyde (Scotland) takes place between May and July (Barnett *et al.*, 1980) whereas in the Ria de Aveiro (Portugal) spawning occurs between March and July (Barroso & Moreira, 1998). The spawning period of several *Nassarius* species from different geographical areas is presented in table 5.1.

Table 5.1 Spawning period of several *Nassarius* species and geographic variations.

Species	**Location**	**Spawning period**	**Reference**
N. incrassatus	Bermuda	All year	Lebour (1945)
N. obsoletus	Massachusetts	March-July	Costello *et al.* (1957)
	North Carolina	March-May	Jenner (1956)
	North Carolina	February - May	Sastry (1971)
N. trivittatus	Massachusetts	May-June	Scheltema & Scheltema (1964)
N. reticulatus	England	February - September	Lebour (1945)
	Plymouth, UK	March-August	Fretter & Graham (1984)
	Eastern English channel	April - August	Fretter & Graham (1984)
	Scotland	May-July	Barnett *et al.* (1980)
	Denmark	April-August	Rasmussen (1973)
	Germany	January-May	Fretter & Graham (1984)
	Portugal	March-July	Barroso & Moreira (1998)

The onset of spawning in *Nassarius reticulatus* is clearly influenced by seawater temperature (Tallmark, 1980). Differences regarding the period of egg capsule deposition between geographically separated populations of *N. obsoletus*, were related to the time when the required environmental temperature to initiate spawning was reached in the various regions (Sastry, 1971). At Hunterston nuclear generating station in the Firth of Clyde, Scotland, spawning occurred in January when the maximum temperature near the warm-water outlet was 12-14°C, whilst 6 km away, where ambient seawater temperatures of 8-9°C occurred, spawning started three months later in May (Barnett, 1972). Barroso & Moreira (1998) found that spawning in the Ria de Aveiro (Portugal) occurred at 11-13.1°C. Spawning in Kvarnbukten Bay (Sweden) started in May at about 11°C, whilst snails from deeper water spawned 3-4 weeks later (Tallmark, 1980). Egg capsules were collected from lake Grevelingen (SW Netherlands) in early April when the water temperatures varied between 6.5 and 9.4°C (Lambeck, 1984). Other spawning cues in prosobranch species, besides temperature, are salinity, lunar periodicity, tidal rhythms and possibly disturbance from surf (Webber, 1977). Fish (1979) suggested a semi-lunar periodicity of spawning in *Littorina littorea* during spring tides at full and new moons. Orton *et al.* (1956) reported that high surf stimulated spawning of *Patella vulgata*, and Tattersall (1920) indicated that *L. littorea* spawned daily at high water.

Several instances of egg capsule production by nassariids during winter have been recorded. Egg production by *Nassarius obsoletus* in North Carolina normally occurs between the end of February and May, although when the seawater temperature is higher than normal some egg capsules can appear from as early as late December (Sastry, 1971). Although spawning of *N. trivittatus* usually takes place at 13°C, between May and June, deposition of egg capsules followed by successful hatching of embryos has been observed in flowing seawater in the laboratory between late October (11.2-8.8°C) and mid December (7.4-3.3°C) (Pechenik, 1978b). Lebour (1931) reported that spawning of *N. reticulatus* in UK (Plymouth) usually starts in March and is finished by the end of September, although egg capsules were recorded throughout the year except during October and November.

Reproductive effort and fecundity of gastropods are affected by food availability (McKillup & Butler, 1979; Cheung & Lam, 1999), salinity (Cheung, 1997) and parental size (Chia & Skeel, 1973; Spight & Emlen, 1976; Chester, 1996). The reproductive output of *Nassarius festivus* was lower in hyposaline environments owing to the limited energy available for reproduction, as a result of the whelks reduced feeding activity and to the increased energy requirements for osmoregulation (Cheung, 1997). Cheung &

Lam (1999) investigated the negative effects of limitations in food supply on egg capsule production by *N. festivus*. McKillup & Butler (1979) reported that fecundity and other reproductive characteristics of *N. pauperatus*, such as egg packaging and size, varied in response to different levels of food availability. Chester (1996) indicated that when adults of the estuarine nudibranch *Tenellia adspersa* were starved for 4 days, they produced significantly fewer spawn masses, with fewer eggs per spawn and eggs of a smaller diameter, than adults which were fed *ad libitum*. Spight & Emlen (1976) noted that when food became more abundant, adult *Thais lamellosa* increased their body size and as a result the size of the average annual clutch also increased, whereas when food was readily available adult of *Thais emarginata* remained about the same size but they increased the frequency of spawning.

The eggs of *Nassarius reticulatus* are packed inside small, flattened capsules, which are often laid on *Zostera*, shells and stones (Lebour, 1931; Fretter & Graham, 1984). Usually many capsules are laid evenly spaced in a row (3 to 15) and clusters of many rows have also been observed (Lebour, 1931; Bouchet *et al.*, 1979). Close packing of gastropod egg capsules traps interstitial water and minimises the total surface area to volume ratio, thus reducing desiccation and the effect of wave impact (Hughes, 1986). When egg capsules are laid on *Zostera* they are often clustered in crevices near the eelgrass roots, and rarely on the leaves (Lebour, 1931). The egg capsules of *N. reticulatus* have a pale horn colour, they are transparent and the eggs are visible through the wall of the capsule (Lebour, 1931). The capsules are vase-shaped in profile, tapering slightly to an apical opening closed by a plug and basally to an expanded attachment disk (Lebour, 1937; Fretter & Graham, 1984). One side of the capsule is almost completely flattened, while the other is slightly convex and the sutures between the two halves run along the sides (Lebour, 1937; Fretter & Graham, 1984; 1994). A rim lies along each edge of the capsule from base to apex and the two unite behind one side of the opening. The capsules have a slanting position with the flat surface uppermost (Lebour, 1931). The walls of *N. reticulatus* capsules have a fibrillar structure but are thinner and more transparent than the capsules of other rachiglossans (Fretter & Graham, 1994).

A detailed description of egg capsule formation and spawning in *Nassarius reticulatus* is provided by Fretter & Graham (1994) and is summarised below. After enrichment of the eggs with layers of albumen inside the female albumen gland, the eggs are transferred to the capsule gland, where ciliary action rotates the egg/albumen mass and creates the preliminary shape of the capsule. Before deposition of the egg capsules the surface of the substratum for egg capsule attachment is investigated by the inhalant siphon and cleaned by the radula. The whelk settles with the right side of the foot raised from the ground so as to form a groove which links the genital aperture with the pedal gland. The egg capsules are passed in an unfinished state from the oviduct, along the temporary groove on the foot, to the pedal gland. The pedal gland grips the capsules so that the plug (opening) is directed towards the inner end. A limited amount of secretion from the gland acts as lubricant and as a hardening mixture. The egg capsule remains inside the capsule gland for a period of 5 minutes during which the longitudinal rims and the basal attachment disk are formed. The capsule is kneaded by the pedal musculature in order to smooth and harden the walls, then the sole of the foot presses the attachment disk against the substratum and the visceral mass is rocked from side to side whilst the base of the egg case is secured. Eventually the anterior part of the foot is drawn up and back, as the muscles of the pedal gland relax, and the capsule is left. The next one is deposited in a similar way nearby so that several come to be arranged in equidistant rows. The egg capsules quickly harden when they come in contact with seawater. Several individuals may use the same spawning ground and lay their egg capsules alongside one another (Fretter & Graham, 1994).

The egg capsules of *Nassarius reticulatus* are 4.5-5 mm in height and about 4 mm across (Lebour, 1931; 1937; Fretter & Graham, 1984). They contain about 50-350 eggs, yellowish or pinkish in colour, 160 um in diameter, each in its own covering but all floating in a common nourishing fluid contained in the capsule (Fretter & Graham, 1984). The dimensions of the egg capsules, the number of eggs per capsule, the size of eggs and the duration of embryonic development, for several *Nassarius* species are summarised in table 5.2. The eggs grow rapidly during embryonic development (Lebour, 1931). Usually all of the embryos of *N. reticulatus* develop as larvae and there are no "nurse" eggs devoured by their neighbours, as in other gastropods such as *Buccinum undatum, Nucella lapillus, Natica catena* and *Rissoa membranacea.* (Lebour, 1931; 1937; Fretter & Graham, 1984). The embryos of *N. reticulatus* hatch 1-2 months after deposition of the egg capsules to release free swimming veliger larvae (Lebour, 1931; Fretter & Graham, 1984). Sometime prior to hatching the larvae can be seen moving inside the capsule with the shell already formed and much yolk inside each

Table 5.2 Summary of a) the height and width (mm) of the egg capsules, b) the number of eggs per capsule and the diameter of the eggs (um) and c) duration of embryonic development of the planktotrophic larvae of several *Nassarius* species.

a

Species	**Capsule height**	**Capsule width**	**Reference**
N. festivus	1.6 mm	1.3 mm	Cheung & Lam (1999)
N. pauperatus	1.1 mm	0.9 mm	McKillup & Butler (1979)
N. trivittatus	2.0 - 4.0 mm	-	Scheltema & Scheltema (1964)
N. incrassatus	1.5 - 2.0 mm	1.3 mm	Fretter & Graham (1984)
N. vibex	1.6 mm	-	Scheltema (1962b)
N. obsoletus	2.7 mm	-	Scheltema (1962b)
N. reticulatus	4.5 - 5.0 mm	4.0 mm	Lebour (1931) Lebour (1937) Fretter & Graham (1984)

b

Species	**Eggs per capsule**	**Diameter of eggs**	**Reference**
N. incrassatus	50-80	-	Lebour (1931)
	50-60	160 um	Fretter & Graham (1984)
N. obsoletus	40-150	160 um	Scheltema (1962b)
	30-300	-	Costello *et al.* (1957)
N. vibex	12-30	-	Scheltema (1962b)
N. trivittatus	42-200	-	Scheltema & Scheltema (1964)
N. reticulatus	126-322	-	Tallmark (1980)
	50-100	160 um	Lebour (1937)
	50-200	-	Ankel (1926)
	50-350	160 um	Fretter & Graham (1984)
N. pygmaeous	40-50	-	Vestergaard (1935)
N. festivus	35	133-140 um	Cheung & Lam (1999)
N. pauperatus	27	143 um	McKillup & Butler (1979)

c

Species	**Embryonic development**	**Reference**
N. incrassatus	2-3 months	Fretter & Graham (1984)
	3-4 weeks	Lebour (1931)
N. reticulatus	1-2 months	Fretter & Graham (1984)
	3-4 weeks	Lebour (1931)
N. obsoletus	4-5 weeks	Costello *et al.* (1957)
N. trivittatus	5-7 days	Scheltema & Scheltema (1964)

larvae (Lebour, 1931). When all the yolk is absorbed the larvae escape from the apical opening of the capsule where a thin covering is broken through (Lebour, 1931). Ankel (1929) first suggested that the larvae secrete a substance which dissolves the plug of the capsule when they are ready to hatch, and later D' Asaro (1969) described the escape of *Nassarius corrugata* larvae from the egg capsule after enzymatic softening of the capsular plug. Many veliger larvae (50-350) hatch from the egg capsules, which have a well formed transparent shell consisting of one whorl and a bilobed velum (Lebour, 1931).

Sandy shore-dwelling gastropods attach their egg capsules onto stable substrata in order to reduce the risk of the egg capsules being transferred up the shore by wave action, where the larvae might die of prolonged exposure (Barnett *et al.*, 1980). However, species of the necklace snails *Natica* deposit their eggs in the interstices of sand grains which are aggregated to form characteristic circular egg collars (Fretter & Graham, 1994). Although these eggs are not anchored to the sand surface their flattened shape reduces the chances of water movements and wave action carrying them away (Fretter & Graham, 1994). Some prosobranch species, such as *Bullia melanoides* produce egg capsules which remain attached onto the foot of the female until they are released (Ansell & Trevallion, 1970). Most sand-dwelling prosobranchs, however, attach their egg capsules onto any available rocks and weeds (Fretter & Graham, 1994). The egg capsules of *Nassarius reticulatus* collected from the shore at Millport, Firth of Clyde, were usually attached to red algae such as *Gigartina stellata* and *Polyides rotundus* growing on the rocks protruding from the sand near the low water mark of spring tides (Barnett *et al.*, 1980). Even in the laboratory *N. reticulatus* showed a tendency to lay egg capsules on available red algae, although when no weed was available they laid their eggs on the sides of the aquarium tanks (Barnett *et al.*, 1980). Barroso & Moreira (1998) also reported that most of the egg capsules of *N. reticulatus* found in the Ria de Aveiro (Portugal) were laid on the red alga *Laurencia pinnatifida*. Deposition of *N. reticulatus* egg capsules on boulders has been recorded only in areas where no other suitable substratum was available (Barnett *et al.*, 1980). Egg capsules of *N. reticulatus* and *N. trivittatus* have also been found attached to the sandy egg collars of *Natica* (Scheltema & Scheltema, 1964; Barnett *et al.*, 1980; Fretter & Graham, 1994).

Strathmann & Chaffee (1984) indicated that when the embryos are embedded in a matrix of jelly, as occurs in many prosobranchs and opisthobranchs, they are spaced out from one another, thus increasing their ventilation and gaseous exchange, which is critical for embryonic survival and development. However, when these gelatinous egg masses are globular in shape the central embryos are less well ventilated and their development is slower than the peripheral ones (Hughes, 1986). The egg capsules produced by many meso- and neogastropods encourage ventilation of the embryos even further than that achieved by jelly masses because the intracapsular fluid can be circulated (Hughes, 1986). The flattened shape of egg capsules, such as the ones deposited by *Nassarius reticulatus*, probably promotes ventilation of all embryos and allows their synchronous development.

The role that the egg capsules play in protecting the embryos against predation, desiccation and salinity stress has being questioned in several studies. The egg capsules are only partially resistant to infections by microrganisms (bacteria, fungi, Protozoa), but they offer protection against predation (Hughes, 1986). For example the egg capsules of *Ilyanassa obsoleta*, which may be accidentally eaten by grazing *Littorina littorea*, resist the snail's digestive enzymes enabling the embryos to survive passage through the gut (Brenchley, 1982). However, significant proportions of dogwhelk egg capsules are damaged by carnivorous predators, such as sea urchins, moon snails and crabs (Spight, 1975a).

Pechenik (1978a) demonstrated that the egg capsules of *Nassarius obsoletus* did not offer the embryos substantial protection against desiccation, so it is unlikely that they evolved as adaptations to intertidal stress. Mortalities of 60% were observed after exposure at 0% relative humidity for 1 hour, and 12% of the capsules failed to hatch after exposure to 75% relative humidity for 3.5 hours (Pechenik, 1978a). Although 0% humidities are unlikely to be encountered in the natural environment, humidities of ~40% are not uncommon in the intertidal zone (Kanwisher, 1957). Advanced-stage embryos are apparently more tolerant of desiccation than early-stage embryos, probably due to the formation of the embryonic shell (Pechenik, 1978a). The egg capsules of the intertidal *N. obsoletus* and the subtidal *N. trivittatus* had similar ability to retain water (Pechenik, 1978a). Pechenik (1978a) suggested that protection of embryos against desiccation depended mostly on adult behaviour during egg laying. The egg capsules of *N. obsoletus* were deposited on the underside of algae and they were kept moist due to the blanketing effect of the seaweed above them (Pechenik, 1978a).

Although egg capsules do not appear to offer substantial protection against predation or desiccation, they can prevent osmotic stress caused by salinity changes to the developing embryos. Pechenik (1982) indicated that immersion of *Ilyanassa obsoleta* embryos, which were removed from their capsules, in seawater of reduced salinity (2-5‰) for as little as 1 hour significantly increased embryonic mortality. Survival of encapsulated embryos, however, was not affected even after exposure for 10 hours to low salinity seawater (2-5‰), although hatching of the larvae was delayed (Pechenik, 1982). Similarly, all the excapsulated embryos of *Nucella lamellosa* died after exposure for 5h to 5‰ salinity, while encapsulated embryos withstood exposures of up to 9h with 0% mortality (Pechenik, 1982). Egg capsule walls are permeable to salts and probably water, thus when the egg capsules were transferred into reduced salinity seawater the osmotic concentration of the intracapsular fluid declined (Pechenik, 1982). The protective role of the capsule for embryos in cases of low salinity stress is actually based on the fact that the capsule reduces the rate of change of osmotic concentration of the intracapsular fluid (Pechenik, 1982). Embryos of *N. lamellosa* subjected to gradual salinity changes were far more tolerant of the stress than individuals subjected to abrupt salinity changes (Pechenik, 1982). The intracapsular fluid appears to consist of a mixture of carbohydrates and proteins, which due to their very low permeability through the capsule wall, may be responsible for the reduction of the rate of osmotic changes inside the capsule (Pechenik, 1982). The rate at which osmotic concentration within the capsules decreases may at least be partly limited by the rate at which the larger sized organic molecules in the intracapsular fluid can diffuse out of the capsules (Pechenik, 1982). Encapsulation of eggs can protect embryos from exposure to environmental stress, thus making worthwhile the high energetic cost for their construction by the female gastropods (Pechenik, 1982).

The aims of the present chapter were: 1) to investigate the spawning behaviour of *Nassarius reticulatus* with particular reference to spawning substrata in the field and in the laboratory, 2) to identify the range of reproductive size classes of adults, 3) to identify the effect of reproductive activity on growth, 4) to estimate reproductive output of different size classes in terms of duration of spawning, number of capsules per female, size of capsules and eggs and number of eggs included per capsule, 5) to investigate the effect of limited food availability on reproductive output, 6) to estimate the duration and the peak of breeding period of whelks held at ambient seawater temperatures under laboratory conditions, 7) to estimate variations in reproductive output between different months within the breeding period and 8) to investigate the effect of temperature on hatching frequency and on the duration of embryonic development.

5.2 MATERIALS AND METHODS

5.2.1 Field sampling

A field survey of the occurrence of the egg capsules of *Nassarius reticulatus* deposited on algal-covered rocks was undertaken in the intertidal lagoon in Rhosneigr during the egg laying season at spring tides in May 2003. The number of egg capsules was counted within 10 replicates of a 50x50 cm metal-frame quadrat and the percentage of algal coverage per quadrat on the rock estimated.

5.2.2 Production of egg capsules and growth rates of different size classes of *Nassarius reticulatus*

Seventy seven *Nassarius reticulatus* that had been collected from the intertidal lagoon in May 2002 (8.9-28.9 mm) and used in the laboratory growth experiments (see chapter 3, section 3.2.3.1), were also used to evaluate the number of egg capsules produced by whelks of a range of different sizes. The individually tagged whelks were divided into groups of 5-6 animals according to their size-class and placed in small plastic tanks. The whelks were separated into ten 2-mm size classes: 8.9-10.9, 11-12.9, 13-14.9, 15-16.9, 17-18.9, 19-20.9, 21-22.9, 23-24.9, 25-26.9, 27-28.9 mm. Details regarding the maintenance and feeding of the whelks during the experiment have been described previously in section 3.2.3.1. The number of egg capsules produced in each tank was recorded weekly between June and July 2002, until egg-capsule production and laying had finished. At the end of the experiment the sex of each individual was identified in order to determine the number of female whelks within each tank and to estimate egg capsule production per female in each size-class (for details of sex identification see section 7.2.3). The mean relative daily growth rate (R%) for all the whelks during the reproductive period (June-July 2002) was calculated for each size class (see chapter 3.2.3.1). Seawater temperature in the small tanks was continuously monitored using a Tinytalk II temperature logger.

Egg capsules were usually laid by *Nassarius reticulatus* on the tank lid or on the walls of the tanks. Capsules were carefully removed from the surfaces of the tanks using a scalpel. Capsules were less frequently laid on small stones that formed part of the shell sand sediment on the base of the tanks, or on empty mussel, *Mytilus edulis*, shells whose flesh had been consumed by the feeding gastropods. Occasionally egg capsules were found on the shell of other living *N. reticulatus*. Additional surfaces (e.g. stones and glass microscope slides covered with strips of vileda scouring pad), that could be easily removed from the tanks were provided to promote egg laying.

5.2.3 The effect of limited food supply on fecundity - Seasonal variation in production of egg capsules

One hundred and forty *Nassarius reticulatus* (20-29.8 mm) were collected from the Rhosneigr intertidal lagoon in March 2004. These were divided into groups of 6-8 animals according to size-class and placed in small plastic tanks. The whelks were separated into nine 1-mm size classes: 20-20.9, 21-21.9, 22-22.9, 23-23.9, 24-24.9, 25-25.9, 26-26.9, 27-27.9, 28-28.9 mm. Details regarding the maintenance and feeding of the whelks during the experiment have been described previously in section 3.2.3.1. There were two replicates of each size class; one replicate was fed with a half freshly opened mussel once a week ("feeding" treatment), whilst the other replicate was fed once a month ("starvation" treatment). The number of egg capsules produced in each tank by the different size classes was recorded weekly for both feeding treatments between March 2004 and April 2005. This experiment was designed to investigate the effects of a limited food supply on the fecundity of *N. reticulatus*.

The dimensions of the newly laid egg capsules (length, width, and weight) were measured, the number of eggs per capsule counted, and the diameter of the eggs measured using a calibrated eyepiece in subsamples of egg capsules (10-40) collected in May 2004 produced by the "fed" and "starved" gastropods from the different size classes. These measurements were defined as the "reproductive output" in the present study. Height was measured from the base of the capsule to the upper edge of the escape zone, and width as the distance across the broadest point. The weight of the egg capsules was measured to the nearest 0.0001 g using a digital balance.

The egg capsules produced by the "fed" *Nassarius reticulatus* of the 25-26.9 mm class between March and November 2004 were used to investigate whether there was a seasonal variation in the capsule dimensions, the number of eggs per capsule and the diameter of the eggs. The "fed" *N. reticulatus* of the 25-26.9 mm size class were selected to investigate seasonal variation in reproductive output because these whelks were producing egg capsules during the whole experimental period.

Twenty egg capsules of known weight were left to hatch in order to estimate the number of larvae released per capsule. Each capsule was placed individually in a 2 litre glass beaker filled with 1800 ml of 0.2 μm filtered ultra-violet irradiated seawater in ambient temperature. On the first day of hatching the larvae were collected using a 63 um plankton mesh sieve and washed back into a 2 litre beaker with 1800 ml of filtered ultra-violet irradiated seawater. The larvae were distributed evenly throughout the water column using the vertical movement of a plunger. Six 1 ml samples were collected using a pipette, and the number of larvae in each 1 ml sample counted under the microscope. The number of larvae estimated in the total volume corresponded to the total number of larvae per egg capsule.

5.2.4 The effect of temperature on the duration of egg capsule hatching

Twenty egg capsules produced on the same day from the spawning *Nassarius reticulatus* held in the laboratory were placed in 2 litre glass beakers filled with 1800 ml of 0.2 μm filtered ultra-violet irradiated seawater. The glass beakers containing the egg capsules were placed in a constant temperature water bath at a range of temperatures from 8.5 to 20°C, aerated and covered with cling film to reduce evaporation. Aeration of the seawater facilitates embryonic development inside the egg capsule by providing more oxygen to the developing embryos and by increasing the rate of diffusion of embryonic wastes out of the egg capsule (see Eyster, 1986). The beakers were checked every 2 days and the number of hatched egg capsules recorded until all of the capsules had hatched. The duration of embryonic development of the egg capsules was estimated for six different temperatures: 20, 17.5, 15, 13, 11 and 8.5°C. Since the adult *N. reticulatus* were spawning in different months and at different ambient seawater temperatures (see results section), the egg capsules used for each one of the six temperature experiments (8.5-20 °C) were laid from the adult whelks in respective ambient temperatures in order to avoid temperature shock.

5.3 RESULTS

5.3.1 Egg capsule production in the intertidal lagoon

Egg capsules of *Nassarius reticulatus* were found during the field survey in the Rhosneigr intertidal lagoon in May 2003. The survey took place during maximum spring tide low water when most of the lagoon area was accessible. Egg capsules were found attached to the stipes of several intertidal macroalgal species. No capsules were found on the fronds of the macroalgae. Egg capsules were attached to macroalgae that were overhanging the sides of the rocks, and not on macroalgae that were located on the upper surfaces of the rocks. As a result, when the capsules are laid they can be carefully covered under the algae where they are kept damp and are protected from predation. *N. reticulatus* generally lays egg capsules in the laboratory whilst in a vertical position.

Figure 5.1 a) Egg capsules of *Nassarius reticulatus* (taken from Bouchet *et al.*, 1979), b) *Chondrus crispus* (taken from www.seaweed.ie ©M. Guiry), c) *Furcellaria lumbricalis* (taken from www.marlin.ac.uk © K. Hiscock) and d) *Plumaria plumosa* (taken from www.algaebase.org © M. Guiry) to illustrate the range of macroalgae found in the Rhosneigr lagoon which had egg capsules of *Nassarius reticulatus* attached.

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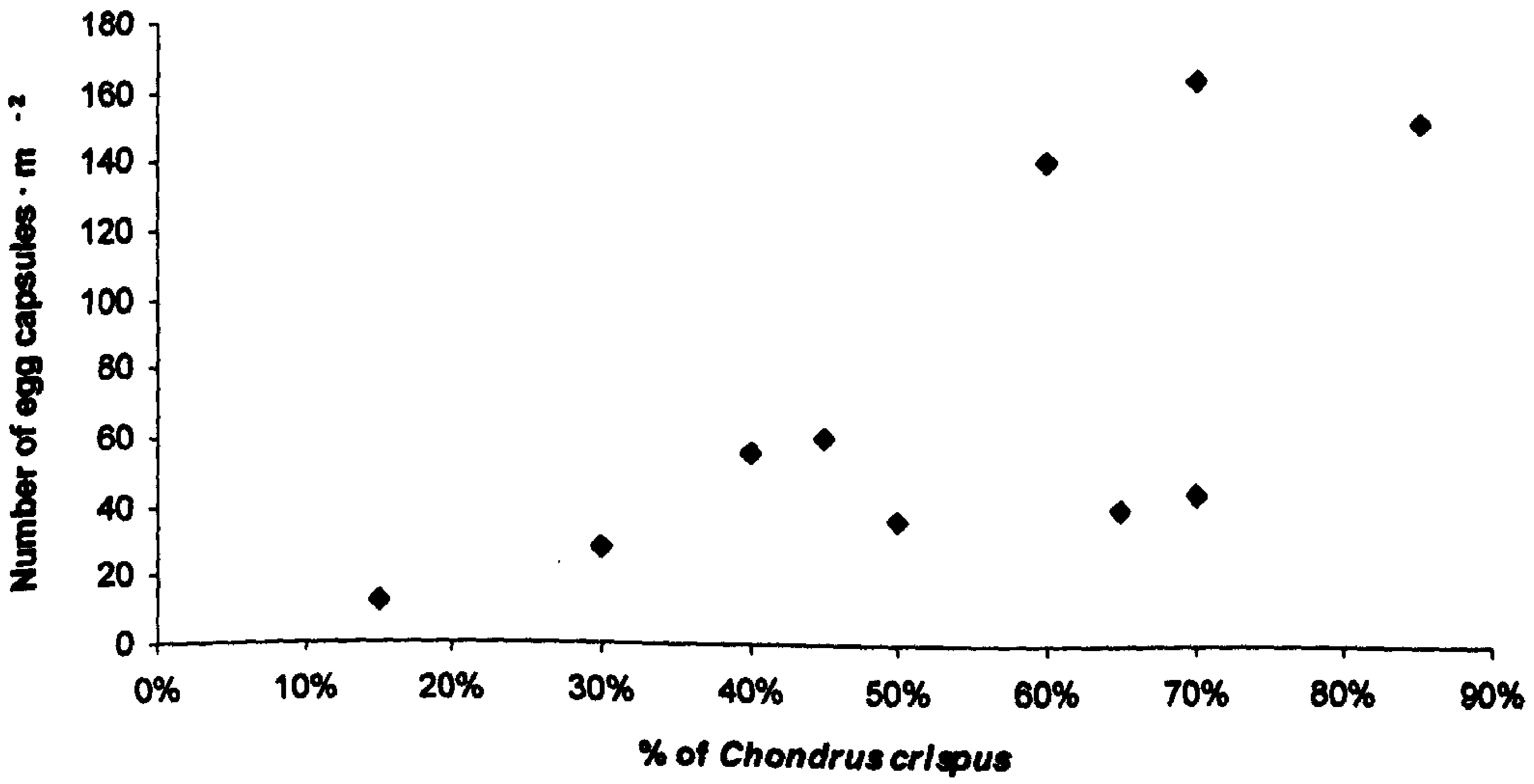
Egg capsules were found attached to 3 different red seaweed species (Rhodophycota, Rhodophyceae): *Chondrus crispus*, *Furcellaria lumbricalis* and *Plumaria plumosa* (figure 5.1). *Chondrus crispus* (Gigartinales, Gigartinaceae) is a widely distributed alga which is commonly found on rocks in the lower intertidal, in rockpools and in the shallow subtidal down to 24 m (Rayment & Pizzola, 2004). It is a small purplish-red seaweed (up to 22 cm long) that has the tendency to turn green in strong sunlight. The fronds are flat and wide with rounded tips, and grow dichotomously from a narrow and unbranched stipe. *Furcellaria lumbricalis* (Gigartinales, Furcellariaceae) grows on rocks and stones in shallow subtidal areas down to 30 m, or more in clear water, and also occurs in rockpools in the eulittoral (Rayment, 2005). It is a reddish brown to brownish black seaweed up to 30 cm long with glossy cylindrical fronds that arise from a highly branched holdfast. *Plumaria plumosa* (previously named *P. elegans*) (Ceramiales, Ceramiaceae) grows on vertical rocks and overhangs in areas of the lower littoral rocky shore (Edwards, 2003). It is a red to brownish-purple seaweed, 5-10 cm long, with compressed thallus and irregularly branched delicate fronds.

The egg caspules of *Nassarius reticulatus* were most commonly attached to *Chondrus. crispus.* Capsules were attached to the other two algal species but only on a few occasions and were never deposited on bare rocky surfaces. A significant correlation (Pearson's r=0.696, p=0.025) was observed between the abundance of *C. crispus* and the presence of egg capsules (figure 5.2).

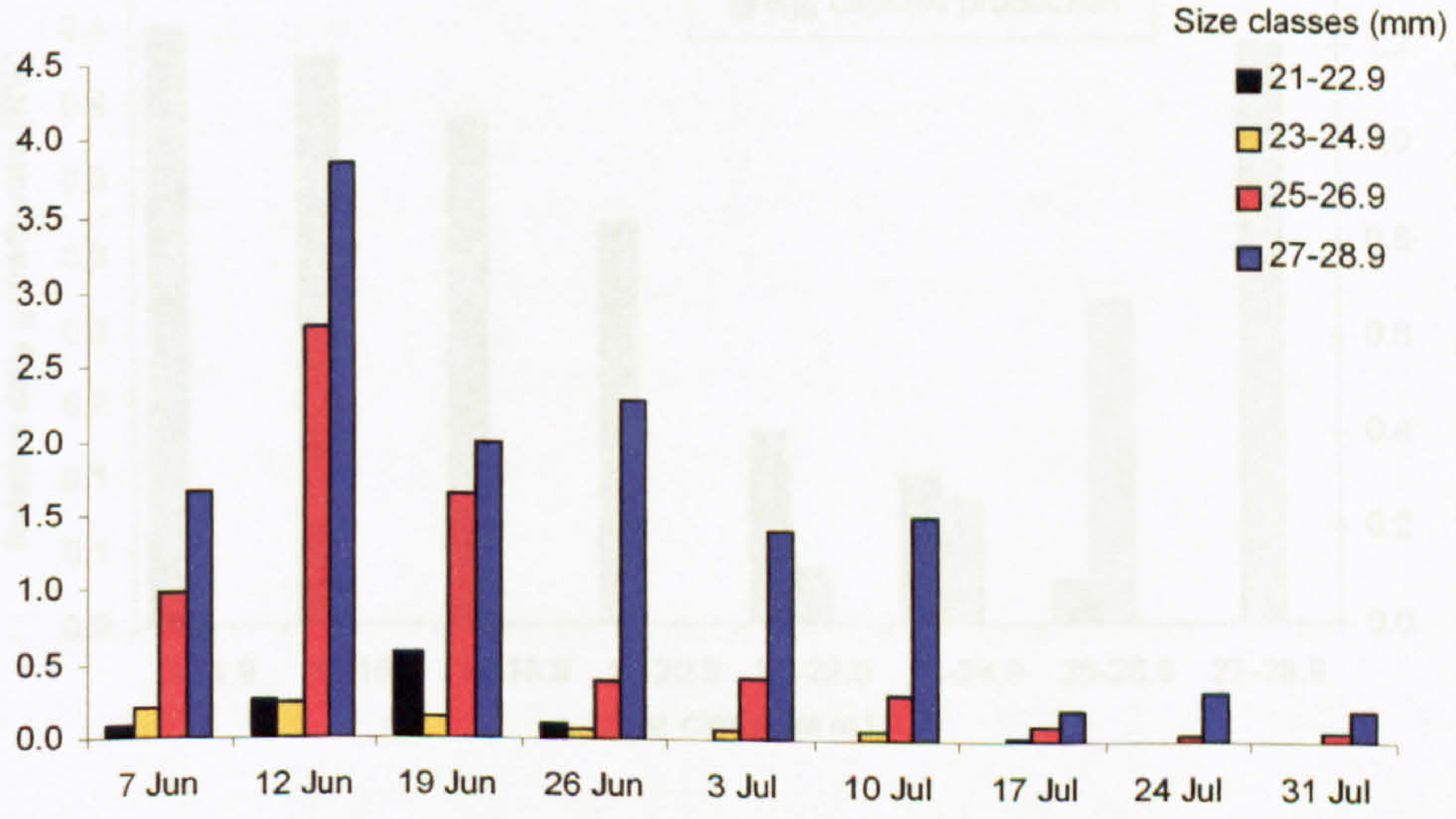
Figure 5.2 Relationship between the number·m^{-2} of *Nassarius reticulatus* egg capsules laid onto the stipes of *Chondrus crispus* in the Rhosneigr lagoon and the percentage cover of *Chondrus crispus*.



5.3.2 Fecundity and growth of different size classes of *Nassarius reticulatus*

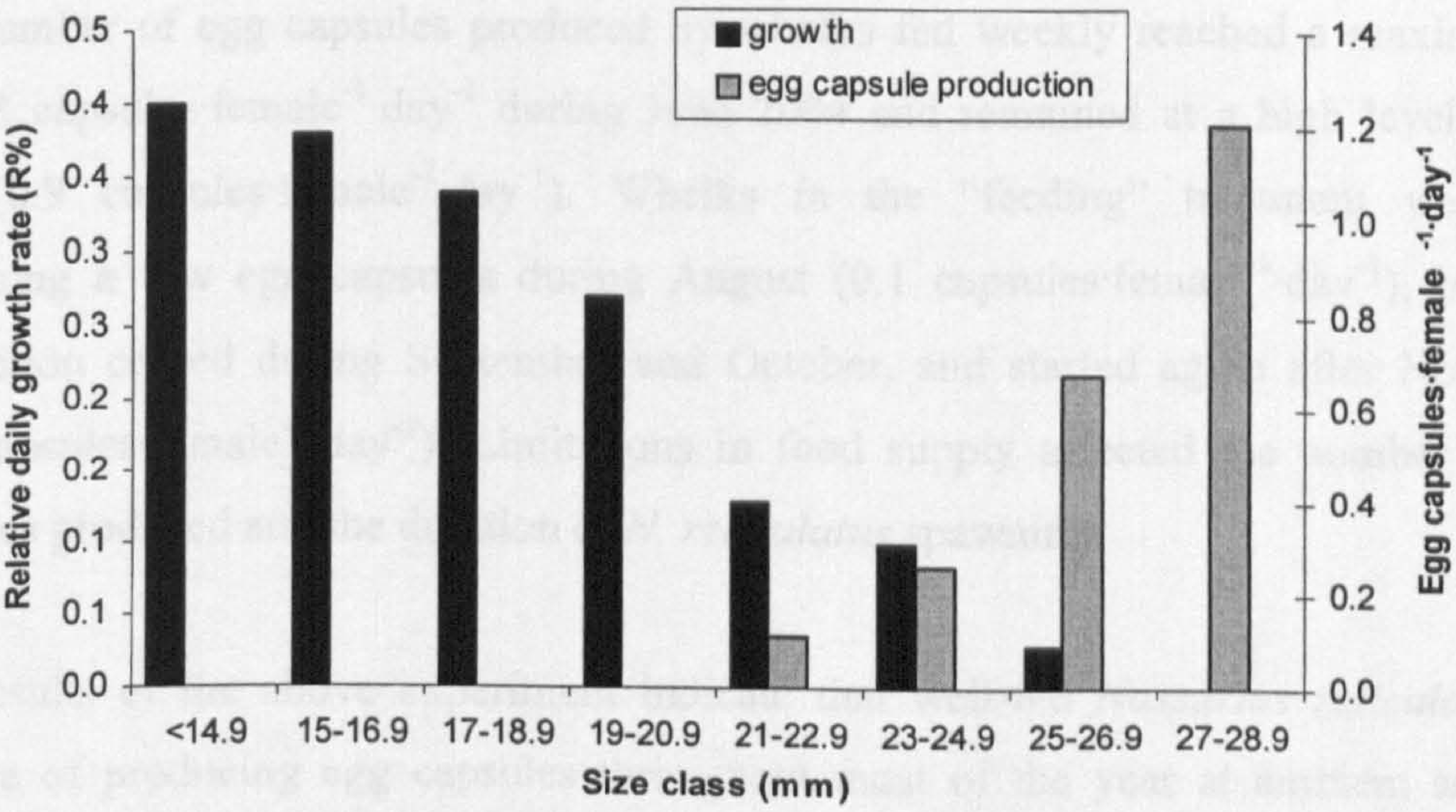
Whelks collected in May 2002 from the intertidal lagoon started laying egg capsules as soon as they were brought into the laboratory. The highest percentage of egg capsules (71.2%) was laid under the tank lid through which the seawater entered the tank. Many capsules (26.9%) were also laid directly onto the aquaria walls, especially in the corners of the tank. Stones, shells of other *Nassarius* and empty mussel shells were less often used as surfaces for egg capsule deposition (0.9%, 0.05% and 0.9% respectively), whilst the glass slides covered with scouring pad rarely promoted egg attachment (0.02%). Fecundity of the different size classes of *N. reticulatus* was estimated as the number of egg capsules $\cdot$female$^{-1}\cdot$day^{-1} produced for the period between June and July 2002 (figure 5.3).

Figure 5.3 Average number of egg capsules$\cdot$female$^{-1}\cdot$day^{-1} produced by *Nassarius reticulatus* from 4 different size classes (mm) between June and July 2002.



No egg capsules were produced from *Nassarius reticulatus* with a shell length smaller than 21 mm. The smaller reproductively active size class of 21-22.9 mm produced <0.6 egg capsules$\cdot$female$^{-1}\cdot$day^{-1} during June and no egg capsules during July. The 23-24.9 mm size class females produced 0.2 capsules$\cdot$female$^{-1}\cdot$day^{-1} during June and only 0.1 capsules$\cdot$ female$^{-1}\cdot$day^{-1} during the beginning of July. The size class of 25-26.9 mm laid >1.0 egg capsules$\cdot$female$^{-1}\cdot$day^{-1} during June, reaching a maximum production of 2.8 capsules$\cdot$female$^{-1}\cdot$day^{-1} in mid June. The 25-26.9 mm sized whelks were still laying egg capsules by the end of July, although production was ~0.4 capsules·female^{-1}·day^{-1} at the beginning of the month and had dropped to ~0.1 capsules·female^{-1}·day^{-1} in late July. The highest number of egg capsules·female^{-1}·day^{-1} was produced by the largest whelks (27-28.9 mm). More than 1.7 capsules·female^{-1}·day^{-1} were produced during June, reaching a maximum of 3.9 capsules·female^{-1}·day^{-1} in mid June. Reproductive activity continued throughout July with a production of about 1.5 capsules·female^{-1}·day^{-1} during the first 10 days of the month, falling to 0.4 capsules·female^{-1}·day^{-1} after mid July. The largest whelks produced the highest numbers of egg capsules and for a longer period of time. The production of egg capsules was highest during mid June for all size classes of whelks.

Figure 5.4 Average relative daily growth rate (R%) and average number of egg capsules produced·female^{-1}·day^{-1} by *Nassarius reticulatus* of different size classes (mm) during the period June to July 2002.



The average relative daily growth rate (R%) of *Nassarius reticulatus* from the different reproductive size classes was calculated from the average increase in shell length during the period June-July 2002 using growth data from the laboratory growth experiments described in section 3.2.3.1 (chapter 3). The total number of egg capsules produced from females of each size class for the period between June and July 2002 was used to estimate the average reproductive activity during the respective months. The relationship between egg capsule production and relative daily growth rate for all size classes of *N. reticulatus* is presented in figure 5.4. Size classes of *N. reticulatus* <20.9

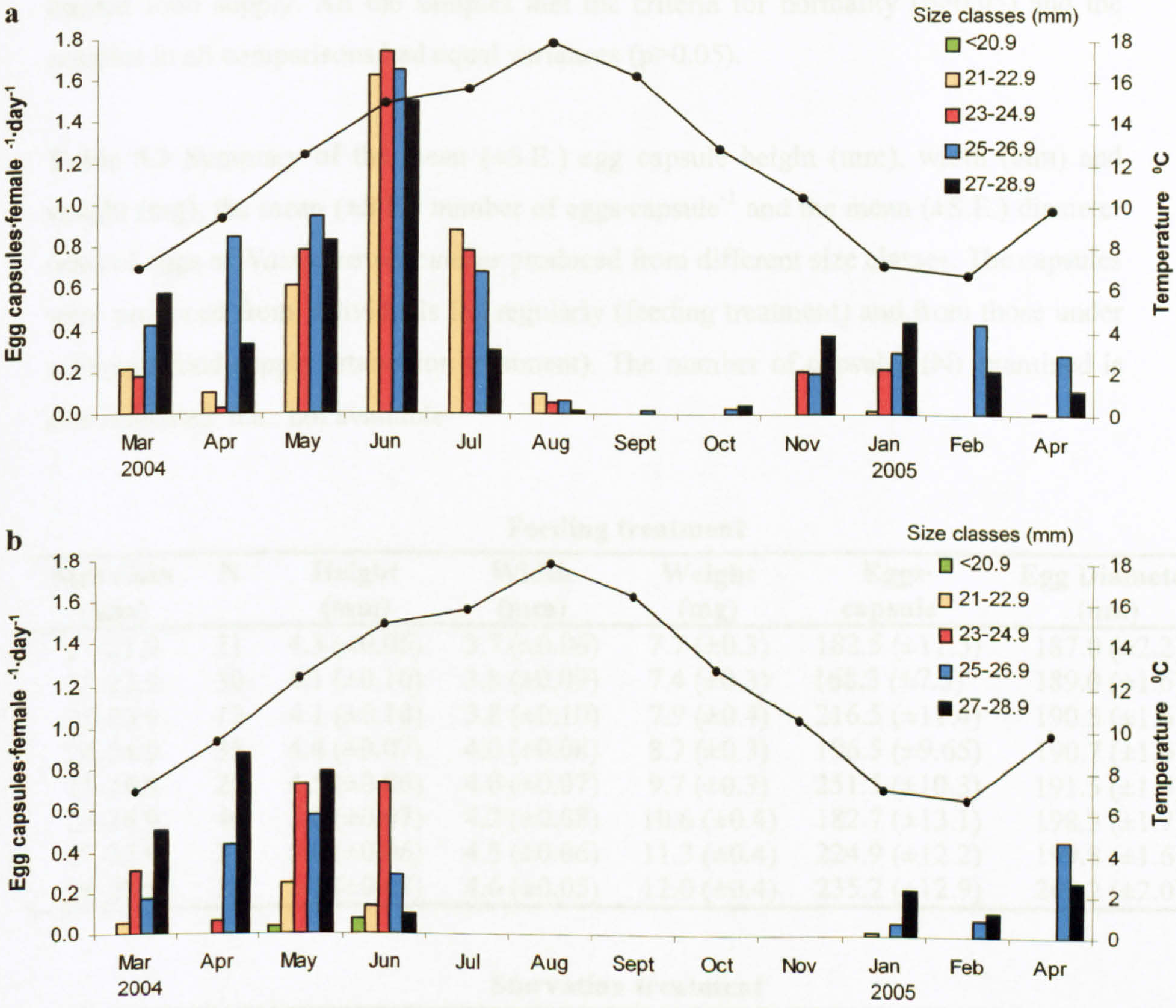
mm grew quickly under the laboratory conditions but did not produce any egg capsules. Larger whelks grew less, and often not at all, yet produced numerous egg capsules. The biggest size class of 27-28.9 mm whelks did not increase in shell length during the experiment but produced the most egg capsules compared to the other size classes.

5.3.3 The effect of limited food supply on the fecundity of *Nassarius reticulatus*

The number of egg capsules produced by each female daily was counted for the two groups of whelks which were fed weekly or monthly in order to investigate the effects of limited food supply on the fecundity of *Nassarius reticulatus* (figure 5.5). Fewer egg capsules were produced by individuals that were maintained under a limited food supply, compared with those fed on a weekly basis. The maximum number of egg capsules produced by the larger size classes in the "starvation" treatment was only 0.7-0.9 capsules$\cdot$female$^{-1}\cdot$day^{-1} between April and June 2004. Egg laying in this group ceased between July 2004 and December 2004, and then commenced in January 2005. The number of egg capsules produced by whelks fed weekly reached a maximum of 1.5-1.8 capsules$\cdot$female$^{-1}\cdot$day^{-1} during June 2004 and remained at a high level during July (0.9 capsules$\cdot$female$^{-1}\cdot$day^{-1}). Whelks in the "feeding" treatment were still producing a few egg capsules during August (0.1 capsules$\cdot$female$^{-1}\cdot$day^{-1}), but then production ceased during September and October, and started again after November (0.4 capsules$\cdot$female$^{-1}\cdot$day^{-1}). Limitations in food supply affected the number of egg capsules produced and the duration of *N. reticulatus* spawning.

The results of the above experiment indicate that well-fed *Nassarius reticulatus* are capable of producing egg capsules throughout most of the year at ambient seawater temperatures under laboratory conditions. More egg capsules were produced as the seawater temperature increased during the early spring and summer, and production reached a peak in June. Although the seawater temperature continued to increase in August, egg capsule production declined rapidly and few capsules were produced in this month. Egg capsule production was very low, but did not cease completely between late summer and autumn. *N. reticulatus* were, however, able to continue laying egg capsules during the winter months when the temperature of the seawater was as low as 7°C between January and February 2005.

Figure 5.5 The average number of egg capsules·female^{-1}·day^{-1} produced by different size classes of *Nassarius reticulatus* between March 2004 and April 2005. a) whelks fed once a week (feeding treatment) and b) whelks fed once a month (starvation treatment). The line represents the seawater temperature inside the experimental tanks.



5.3.4 Variation in the dimensions of the egg capsules, the number of eggs per capsule and the size of eggs of *Nassarius reticulatus*

5.3.4.1 Differences between size classes

A summary of the dimensions (height and width) and weight of the egg capsules, the number of eggs per capsule and the diameter of the eggs from sub-samples of egg capsules collected during May 2004 that were produced from whelks of different size classes is presented in table 5.3 and illustrated in figures 5.6 ("fed") and 5.7 ("starved"). A One-Way ANOVA was conducted in order to compare the size and weight of the capsules, as well as the number and size of the eggs per capsule, between egg capsules that were produced from different size classes of individuals. The data were analysed separately for regularly fed *Nassarius reticulatus* and for whelks provided with a limited food supply. All the samples met the criteria for normality ($p>0.05$) and the samples in all comparisons had equal variances ($p>0.05$).

Table 5.3 Summary of the mean (±S.E.) egg capsule height (mm), width (mm) and weight (mg), the mean (±S.E.) number of eggs$\cdot$capsule^{-1} and the mean (±S.E.) diameter (um) of eggs of *Nassarius reticulatus* produced from different size classes. The capsules were produced from individuals fed regularly (feeding treatment) and from those under a limited food supply (starvation treatment). The number of capsules (N) examined is also indicated. n.a.: not available

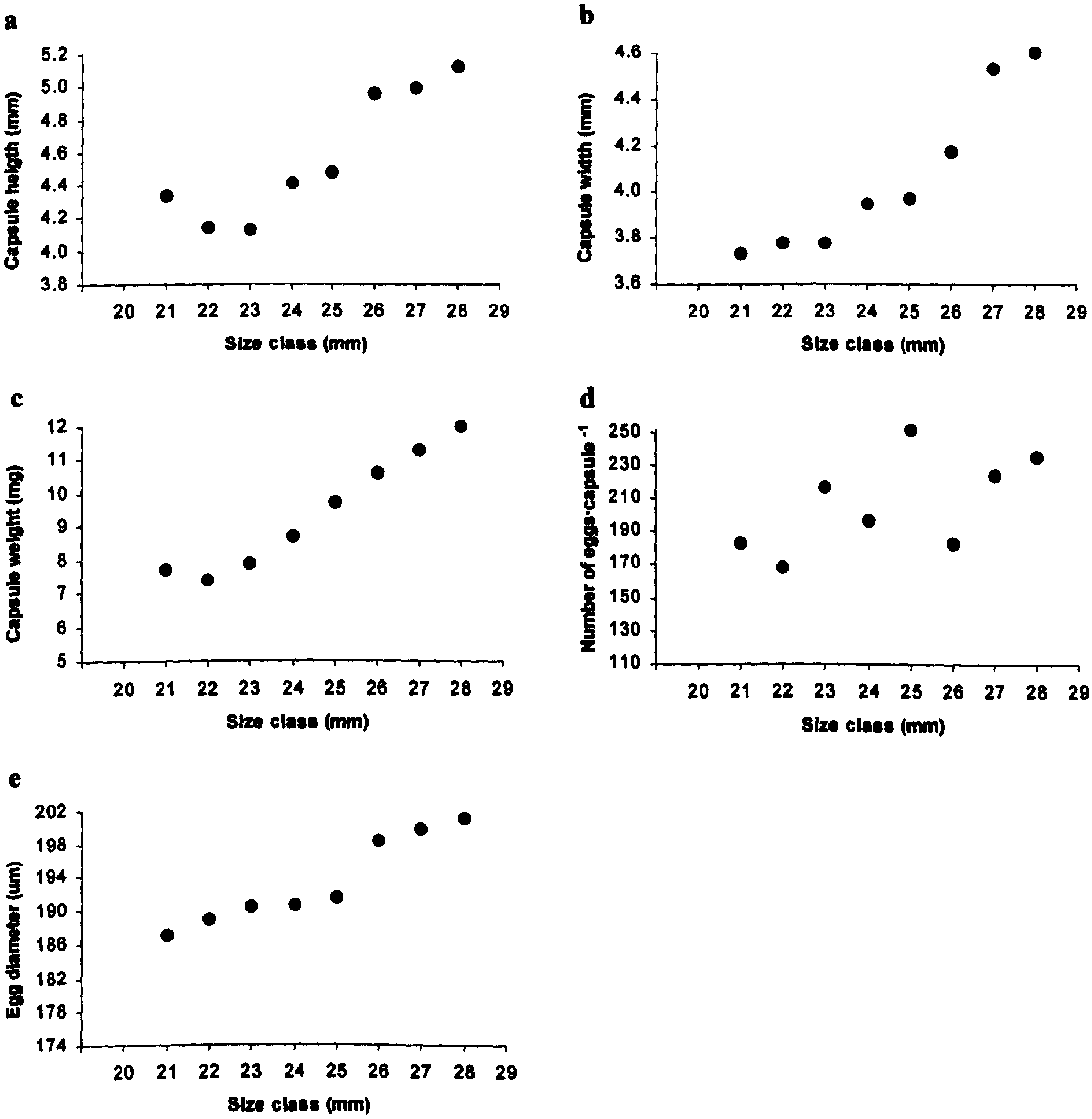
Feeding treatment

Size class (mm)	N	Height (mm)	Width (mm)	Weight (mg)	Eggs$\cdot$capsule^{-1}	Egg Diameter (um)
21-21.9	21	4.3 (±0.05)	3.7 (±0.06)	7.7 (±0.3)	182.5 (±11.5)	187.0 (±2.2)
22-22.9	30	4.1 (±0.10)	3.8 (±0.09)	7.4 (±0.3)	168.3 (±7.5)	189.0 (±1.6)
23-23.9	13	4.1 (±0.10)	3.8 (±0.10)	7.9 (±0.4)	216.5 (±11.4)	190.5 (±1.6)
24-24.9	35	4.4 (±0.07)	4.0 (±0.08)	8.7 (±0.3)	196.5 (±9.65)	190.7 (±1.6)
25-25.9	26	4.5 (±0.06)	4.0 (±0.07)	9.7 (±0.3)	251.5 (±10.3)	191.5 (±1.6)
26-26.9	40	5.0 (±0.07)	4.2 (±0.08)	10.6 (±0.4)	182.7 (±13.1)	198.3 (±1.7)
27-27.9	31	5.0 (±0.06)	4.5 (±0.06)	11.3 (±0.4)	224.9 (±12.2)	199.8 (±1.6)
28-28.9	34	5.1 (±0.07)	4.6 (±0.05)	12.0 (±0.4)	235.2 (±12.9)	201.2 (±2.0)

Starvation treatment

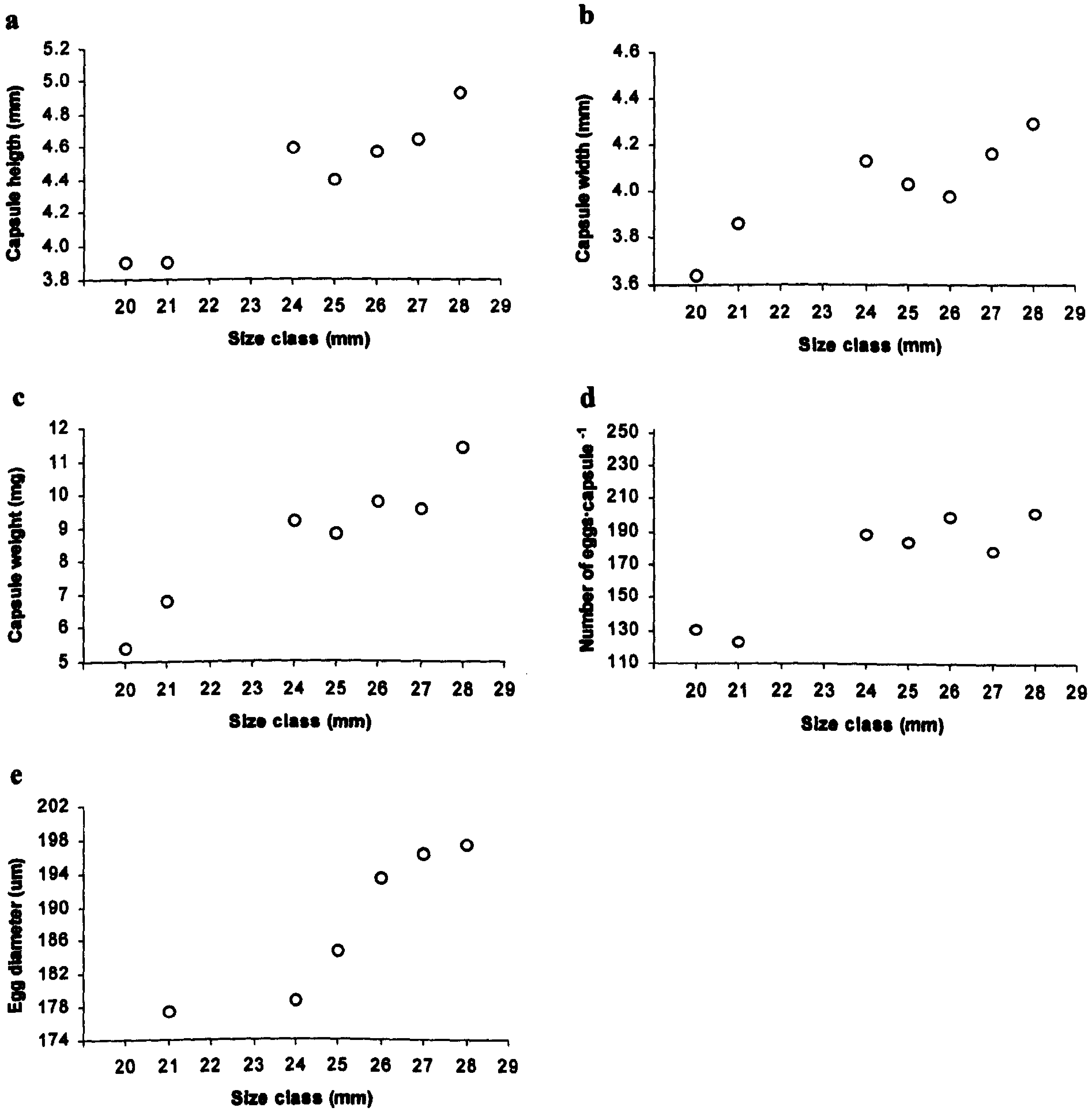
Size class (mm)	N	Height (mm)	Width (mm)	Weight (mg)	Eggs$\cdot$capsule^{-1}	Egg Diameter (um)
20-20.9	3	3.9 (±0.18)	3.6 (±0.10)	5.4 (±0.2)	129.3 (±8.0)	n.a.
21-21.9	12	4.0 (±0.10)	3.9 (±0.09)	6.8 (±0.3)	122.4 (±14.5)	177.4 (±4.0)
24-24.9	27	4.6 (±0.08)	4.1 (±0.06)	9.2 (±0.3)	187.4 (±13.2)	178.6 (±3.4)
25-25.9	23	4.4 (±0.07)	4.0 (±0.07)	8.8 (±0.3)	183.2 (±11.2)	184.6 (±2.2)
26-26.9	30	4.6 (±0.08)	4.0 (±0.08)	9.8 (±0.4)	198.1 (±11.6)	193.4 (±1.9)
27-27.9	29	4.7 (±0.06)	4.2 (±0.07)	9.6 (±0.4)	177.5 (±10.2)	196.3 (±2.3)
28-28.9	20	4.9 (±0.07)	4.3 (±0.04)	11.4 (±0.4)	200.4 (±18.8)	197.3 (± 1.3)

Figure 5.6 Mean values of a) height of egg capsules (mm), b) width of egg capsules (mm), c) weight of egg capsules (mg), d) number of eggs·capsule^{-1} and e) diameter of eggs (um) produced from all the different size classes of regularly fed *Nassarius reticulatus*.



Significant differences were found in the height, width, weight of egg capsules, number of eggs per capsule and diameter of eggs that were produced from whelks of different size classes that were fed regularly ("fed") (figure 5.6). The capsules from the "fed" whelks from the larger size classes had a significantly greater height, width and weight than those from the smaller size classes of "fed" whelks (F=27.21, p<0.001 for height,

Figure 5.7 Mean values of a) height of egg capsules (mm), b) width of egg capsules (mm), c) weight of egg capsules (mg), d) number of eggs·capsule^{-1} and e) diameter of eggs (um) produced from all the different size classes of *Nassarius reticulatus* provided with limited food supply.



F=20.66, P<0.001 for width, and F=24.75, p<0.001 for weight). In addition, the larger "fed" whelks produced egg capsules that contained significantly more eggs per capsule (F=6.36, p<0.001) and the diameter of the eggs was significantly bigger (F=7.96, p<0.001) than those produced by the smaller whelks. Similar results were obtained from whelks that were provided with a limited food supply ("starved") (figure 5.7). Larger whelks produced egg capsules that had a significantly larger height, width and weight than those produced by the smaller size classes of "starved" whelks (F=10.28, p<0.001 for height, F=3.96, P<0.001 for width, and F=12.51, p<0.001 for weight). The larger size classes of "starved" whelks produced egg capsules with significantly more eggs per capsule (F=2.55, p<0.001) and the diameter of the eggs was significantly larger (F=10.71, p<0.001).

5.3.4.2 Differences between feeding regimes

A Two-Sample t-test was used to compare the size (height, width) and weight of the capsules, as well as the number and diameter of eggs per capsule (measurements in table 5.3), produced by regularly fed whelks or by those under a limited food supply. Comparisons were made between similar size classes of "fed" and "starved" gastropods and they are summarised in table 5.4 All the samples met the criteria of normality (p>0.05) and the samples in all comparisons had equal variances (p>0.05).

Table 5.4 Summary of 2-sample t-test comparisons (t-value and associated probability) between the egg capsules produced from respective size classes of "fed" and "starved" whelks. The height, width and weight of capsules, the number of eggs·capsule^{-1} and the diameter of the eggs were compared. * indicates where the egg capsules from the fed whelks are significantly larger and the eggs are more numerous and larger (p<0.05).

Size class	21-21.9 mm	24-24.9 mm	25-25.9 mm
Height	T= 2.89, p= 0.011 *	T= -1.60, p= 0.116	T= 0.82, p= 0.419
Width	T= -1.19, p= 0.249	T= -1.97, p= 0.055	T= -0.66, p= 0.515
Weight	T= 2.26, p= 0.034 *	T= -1.16, p= 0.250	T= 2.08, p= 0.044 *
Eggs/capsule	T= 2.11, p= 0.042 *	T= 3.23, p= 0.002 *	T= 2.57, p= 0.012 *
Egg diameter	T= 3.25, p= 0.006 *	T= 0.55, p= 0.582	T= 4.50, p< 0.001 *

Size class	26-26.9 mm	27-27.9 mm	28-28.9 mm
Height	T= 3.84, p< 0.001 *	T= 3.99, p< 0.001 *	T= 1.83, p= 0.074
Width	T= 1.74, p= 0.087	T= 4.06, p< 0.001 *	T= 4.44, p< 0.001 *
Weight	T= 1.46, p= 0.149	T= 3.39, p= 0.001 *	T= 1.01, p= 0.319
Eggs/capsule	T= 1.96, p= 0.053	T= 1.29, p= 0.200	T= 1.64, p= 0.104
Egg diameter	T= -0.88, p= 0.384	T= 2.98, p= 0.004 *	T= 1.53, p= 0.139

Generally *Nassarius reticulatus* which had a limited food supply constructed smaller egg capsules and contained fewer eggs of a smaller size per capsule, compared with regularly fed whelks (see table 5.3). The t-test comparisons (table 5.4) confirmed that on several occasions the size of capsules, the egg diameter and the number of eggs per capsule were significantly different between fed and "starved" whelks.

5.3.4.3 Seasonal differences

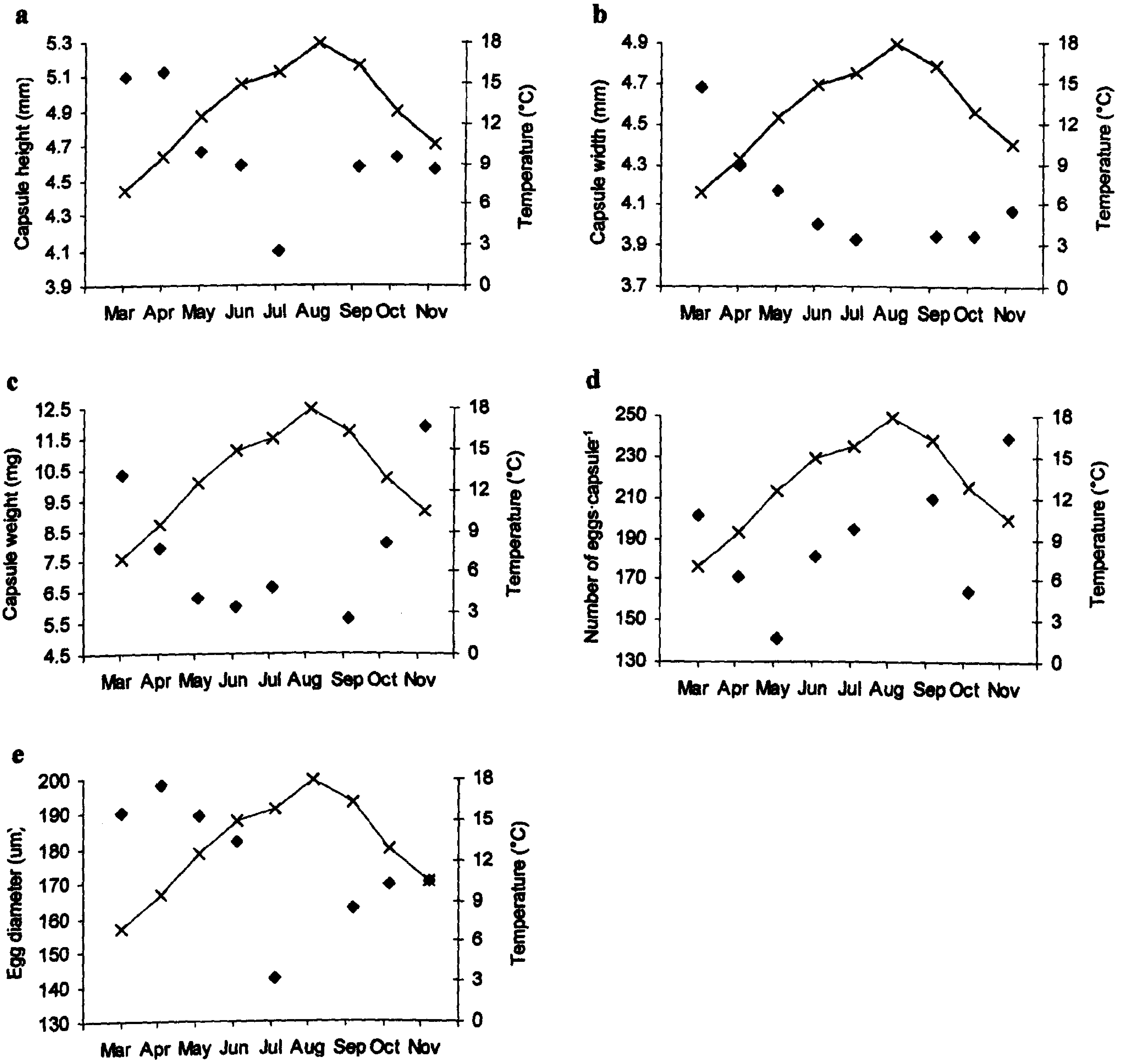
Sub-samples of egg capsules produced monthly from 25-26.9 mm *Nassarius reticulatus* that were regularly fed and were reproductively active during the period from March to November 2004 were examined. Smaller size classes from the "feeding treatment" or whelks from the "starvation" treatment did not produce egg capsules during the autumn and winter and were therefore not considered further. A One-Way ANOVA was conducted to compare the size and weight of the capsules, as well as the number and size of eggs per capsule, between the different months (table 5.5). All the samples met the criteria of normality ($p>0.05$) and the samples in all comparisons had equal variances ($p>0.05$).

Table 5.5 Summary of the mean (±S.E.) height (mm), width (mm) and weight (mg) of egg capsules, the number of eggs·capsule^{-1} and the egg diameter (um) produced by *Nassarius reticulatus* in different months. The size of the egg capsule samples studied (N) is shown.

Month	**N**	**Height (mm)**	**Width (mm)**	**Weight (mg)**	**Eggs per capsule**	**Diameter (um)**
March	20	5.1 (±0.12)	4.7 (±0.11)	10.3 (±0.5)	201.5 (±17.5)	190.3 (±8.0)
April	14	5.1 (±0.14)	4.3 (±0.12)	7.9 (±0.4)	171.5 (±24.3)	198.2 (±3.0)
May	22	4.7 (±0.09)	4.2 (±0.08)	6.3 (±0.5)	140.8 (±23.3)	189.3 (±2.5)
June	15	4.6 (±0.11)	4.0 (±0.09)	6.0 (±0.4)	181.2 (±21.9)	182.0 (±2.2)
July	15	4.1 (±0.11)	3.9 (±0.08)	6.6 (±0.3)	194.6 (±14.3)	142.0 (±2.9)
September	15	4.6 (±0.11)	3.9 (±0.09)	5.6 (±0.3)	209.6 (±13.0)	163.0 (±1.6)
October	20	4.6 (±0.04)	3.9 (±0.05)	8.1 (±0.5)	164.8 (±22.1)	169.5 (±1.6)
November	15	4.6 (±0.06)	4.1 (±0.06)	11.9 (±0.4)	239.4 (±43.3)	170.8 (±1.5)

Significant differences were found in the size (height and width) and weight of egg capsules produced by *Nassarius reticulatus* between March and November (figure 5.8). The height of the capsules produced during the summer months was significantly smaller ($F=9.93$, $p<0.001$) than the height of capsules produced during the winter and early spring months. Similarly, the width and weight of the capsules produced during the summer and early autumn months was significantly smaller than the width and

Figure 5.8 Monthly variation in a) egg capsule height (mm), b) egg capsule width (mm), c) egg capsule weight (mg), d) the number of eggs·capsule^{-1} and e) egg diameter (um) produced by regularly fed *Nassarius reticulatus* (25-26.9 mm). The line represents the seawater temperature (°C) inside the experimental tanks.



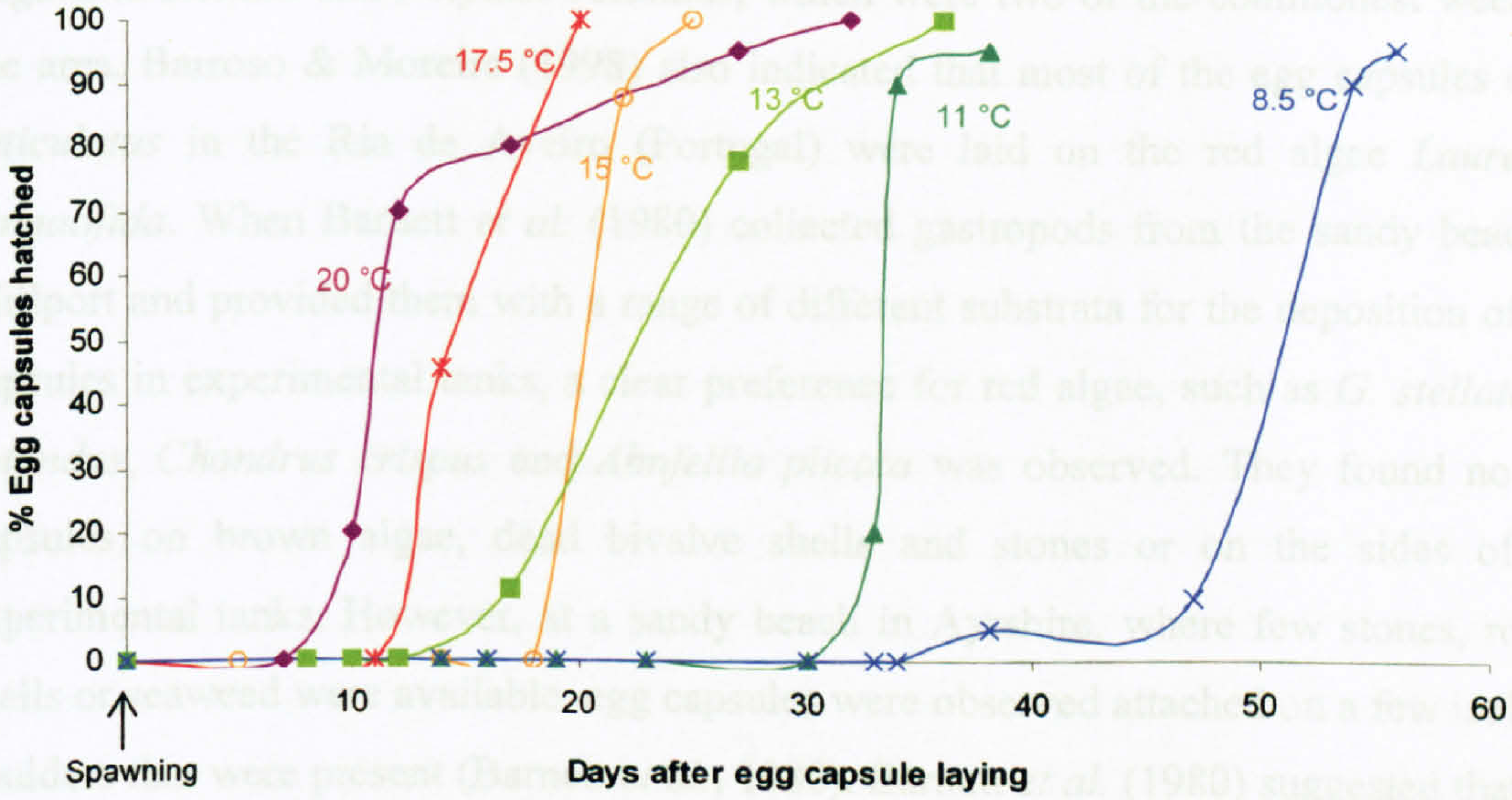
weight of capsules produced during the winter and early spring months (F=9.96, p<0.001 for width and F=25.61, p<0.001 for weight). Although the diameter of the eggs contained within the capsules was significantly smaller during summer (F=30.34, p<0.001), there was no significant difference in the number of eggs per capsule during the different months (F=1.09, p=0.385). Since *N. reticulatus* produced similar number of eggs throughout the year but smaller and lighter weight capsules during the summer, then in order to maintain a constant number of eggs per capsule, the diameter of the eggs within the capsules had by necessity to become smaller. Thus light weight capsules produced during the summer contained similar numbers of eggs to those in the heavier winter egg capsules. No significant correlation was found between the number of eggs per capsule and the weight of the capsule (Pearson's correlation: r= -0.046, p=0.751). Accordingly, when egg capsules of known weight were allowed to hatch, the number of larvae released per capsule was not significantly correlated with the weight of the capsule (Pearson's correlation: r=0.033, p=0.907).

5.3.5 The effect of temperature on the duration of egg capsule hatching

Figure 5.9 shows the percentage of hatched egg capsules in relation to the time since egg capsule laying for 6 different temperatures, 20, 17.5, 15, 13, 11 and 8.5°C. Egg capsules incubated at 20°C started hatching 10 days after spawning, 80% of the capsules had hatched after 17 days, and all the capsules had hatched after 32 days. Egg capsules at 17.5 and 15°C started hatching after 14 and 22 days respectively, and all capsules had hatched by day 20 and 25 respectively. Although the higher temperature of 20°C accelerated the initiation of larval release from the egg capsules, hatching of all the egg capsules incubated at 17.5 and 15°C was completed in a shorter period of time than capsules incubated at 20°C. Of particular note is the fact that seawater temperatures as high as 20°C were recorded in the intertidal lagoon (see chapter 3, figure 3.4) during July and August, at a time when egg capsule laying was reduced in the laboratory. During the peak reproductive period (May-June) the seawater temperature ranged from 15°C to 18°C which coincided with the shortest period of embryonic development in the laboratory.

The larvae in the egg capsules incubated at 13 and 11°C began hatching 17 and 33 days respectively after spawning, and all the capsules had hatched after 36 and 38 days respectively. Larvae from egg capsules maintained at 8.5°C started hatching 38 days after egg capsule laying and 95% of the capsules had hatched after 56 days. This study demonstrated that larvae were released successfully from egg capsules even at low winter temperatures. Since spawning of the larger *Nassarius reticulatus* takes part during most months throughout the year (see figure 5.5 a), release of larvae from the egg capsules is obviously possible under a range of different seawater temperatures, although embryonic development takes longer at lower temperatures.

Figure 5.9 The percentage of egg capsules hatched in relation to the time after egg capsule laying. The egg capsules were incubated at 6 different temperatures (°C) which are represented with different colours on the figure.



5.4. DISCUSSION

Egg capsules of *Nassarius reticulatus* in Rhosneigr intertidal lagoon were found attached to the stipes of the red seaweeds *Chondrus crispus*, *Furcelaria lumbricalis* and *Plumaria plumosa*. All three red algae are commonly found on rocks, overhangs and rockpools in the lower intertidal and shallow subtidal areas of the lagoon. Egg caspules were mainly laid on *C. crispus*, occasionally on *F. lumbricalis* and *P. plumosa*, but were never found attached to bare rock surfaces. *C. crispus* was highly abundant in the intertidal lagoon and its solid, narrow and unbranched stripe offered an ideal substratum for egg capsule attachment. The egg caspules were deposited on the stipes and not on the fronds of the red algae. Pechenik (1978a) reported that egg capsules of *N. obsoletus* were mostly deposited on the holdfast of algae, in order to maximise the contact stimulation of the foot during deposition. The egg capsules of *N. reticulatus* were usually laid on the underside of the stipes of the red algae that were overhanging from the sides of the rocks in the lagoon, thus promoting coverage of the egg capsules by the algal fronds and protection against desiccation and predation. Pechenik (1978a) indicated that the egg capsules of *N. obsoletus* were also deposited on the undersides of algae, where most of them were at least partially protected from exposure to air of low humidity and only <8% were entirely exposed during low tide.

Barnett *et al.* (1980) reported that *Nassarius reticulatus* from a sandy beach at Millport (Firth of Clyde, Scotland), deposited most of their egg capsules on red algae such as *Gigartina stellata* and *Polyides rotundus*, which were two of the commonest weeds in the area. Barroso & Moreira (1998) also indicated that most of the egg capsules of *N. reticulatus* in the Ria de Aveiro (Portugal) were laid on the red algae *Laurencia pinnatifida*. When Barnett *et al.* (1980) collected gastropods from the sandy beach in Millport and provided them with a range of different substrata for the deposition of egg capsules in experimental tanks, a clear preference for red algae, such as *G. stellata*, *P. rotundus*, *Chondrus crispus* and *Ahnfeltia plicata* was observed. They found no egg capsules on brown algae, dead bivalve shells and stones or on the sides of the experimental tanks. However, at a sandy beach in Ayrshire, where few stones, rocks, shells or seaweed were available, egg capsules were observed attached on a few isolated boulders that were present (Barnett *et al.*, 1980). Barnett *et al.* (1980) suggested that red algae are a more favourable substrate for egg deposition than brown algae, because they are covered by a much thinner surface of mucilage, which is absent from the older parts. *N. reticulatus* are able to clean the surface of the substratum for egg capsule attachment with their radula, though a thicker layer of mucilage might constrain egg capsule deposition (Barnett *et al.*, 1980). Adhesion of the basal disk of the egg capsules onto hard surfaces of rocks and stones is probably easier; however, whelks appear to prefer attachment of egg capsules onto seaweed because their fronds offer efficient camouflage and higher protection against predation by sea urchins (e.g. *Psammechinus* and *Echinus*) feeding on rock-encrusting organisms (Barnett *et al.*, 1980). Seaweeds tend to attach onto larger and more stable stones and rocks, which are less likely to be moved by wave action, whereas small stones are liable to be covered by sand during storms and any attached capsules may be smothered (Barnett *et al.*, 1980). The fronds of seaweed offer the capsules protection against wave action and turbulence, whilst during exposure at spring low tide the capsules receive protection from desiccation and high air temperatures (Barnett *et al.*, 1980).

During the survey in the Rhosneigr intertidal lagoon most *Nassarius reticulatus* egg capsules were laid on the stipes of *Chondrus crispus*. Selection of one algal species in favour of others maybe a combination of several factors, such as suitable shape or surface. For example Barnett *et al.* (1980) suggested that *Gigartina stellata* and *Polyides rotundus* were more often selected for egg capsule deposition, because they have leathery and narrow stipes, around which the basal anchoring part of the egg capsule can be easily wrapped. The fine thread-like fronds of *Ahnfeltia plicata* or the tubular membranaceous thallus of *Dumontia incrassata* provided a very poor surface for attachment of egg capsules (Barnett *et al.*, 1980). *C. crispus* has a leathery narrow stipe, which provides a non-slippery and firm surface for egg capsule attachment, in contrast to the filamentous thallus of *Plumaria plumosa* which rarely had egg capsules attached.

In the laboratory tanks, where no algae were present, *Nassarius reticulatus* successfully laid egg capsules on the tank surfaces. Most egg capsules were deposited under the slots of the tank lid through which the seawater circulated. Egg capsules were also laid on the vertical walls of the plastic tanks, and especially along the corners of the tank walls. Pechenik (1978a) indicated that *N. obsoletus* laid egg capsules on parts of the experimental tanks that were maximising the contact stimulation of the female foot during deposition (low thigmokinetic adult behaviour), such as along and under the curled edges of the tanks. From the positioning of the egg capsules under the tank lid or on the tank walls it can be concluded that female *N. reticulatus* are able to spawn whilst hanging in a vertical position. *N. obsoletus* similarly prefers to lay its egg capsules whilst hanging vertically (Pechenik, 1978a).

This study has shown that *Nassarius reticulatus* first spawns in the laboratory at a size of 21 mm. Gastropods smaller than 20.9 mm did not produce any egg capsules in the laboratory but grew faster than the larger size classes. Larger whelks (25-28.9 mm) produced more egg capsules$\cdot$female$^{-1}\cdot$day^{-1} and spawning occurred for a longer period of time. Egg capsule production of the smaller whelks (21-24.9 mm) ceased after the end of June, whilst the larger whelks (25-28.9 mm) continued producing egg capsules until the end of July. The larger whelks grew very little (25-26.9 mm) or not at all (27-28.9 mm) during the reproductive period presumably since they had reached their asymptotic maximum size (see chapter 3). The smaller size classes of *N. reticulatus* invested most of their assimilated energy into growth, while bigger size classes invested most of their assimilated energy into reproduction.

Cheung & Lam (1999) reported that during the reproductive period, *Nassarius festivus* did not increase in size and suggested that the bulk of energy gained by feeding was channelled into reproduction rather than shell growth. Similar results have been reported for other gastropod species. For example the number of eggs produced by the estuarine nudibranch *Tenellia adspersa* was greater as adult size increased, whilst growth rates declined (Chester, 1996). Similarly the saccoglossan opisthobranch *Olea hansineensis*, prior to maturity directed most of its assimilated energy into body and shell growth; however, following maturity the majority of energy was invested in reproduction (Chia & Skeel, 1973). Spight & Emlen (1976) reported that body size in adult *Thais lamellosa* and *Thais emarginata* was directly proportional to the size of the egg clutch produced. Roberts & Hughes (1980) indicated that fecundity of *Littorina rudis* increased with increasing parental size. Changes in fecundity with size are often associated with an increase in gonad volume as the animal grows (Hughes, 1986).

The egg capsules of *Nassarius reticulatus* produced by the largest females were greater in size (height, width), heavier, and contained more eggs of a greater diameter than capsules produced by smaller whelks. Similar observations were obtained from the egg capsules produced either from regularly fed individuals or from those under limited food supply. Webber (1977) indicated that the number of eggs per capsule in a given species may vary due to the size of the adult. Scheltema & Scheltema (1964) reported that egg capsule height of *N. trivittatus* ranged from 2 to 4 mm, and they suggested that larger females produced egg capsules of a greater size than small ones. Scheltema & Scheltema (1964) further demonstrated that the number of eggs per capsule in *N. trivittatus* was related to the size of the capsule with larger egg capsules containing more eggs.

The availability of carrion for scavengers, such as *Nassarius reticulatus*, may be ephemeral and spatially unpredictable. Although scavengers may experience times when carrion is abundant, they may also experience unpredictable episodes of food shortage (McKillup & McKillup, 1997). Indirect evidence by McKillup & Butler (1979) suggested that nassariid gastropods may frequently be subject to limited food supplies in the field. The supply of carrion may be an important constraint on reproduction of both obligate and facultative scavengers (McKillup & McKillup, 1997). Limitations in food supply clearly had a negative effect on fecundity and duration of spawning by *N. reticulatus* in this study. Fewer egg capsules (maximum: 0.7-0.9 capsules$\cdot$female$^{-1}\cdot$day^{-1}) were produced when whelks were maintained under a limited food supply, compared with those fed on a regular basis (maximum: 1.5-1.8 caspules$\cdot$female$^{-1}\cdot$day^{-1}). In addition, the spawning period for regularly fed whelks ceased between September and October, while those held under a limited food supply did not produce any egg capsules between July and January of the following year. Egg capsules produced by *N. reticulatus* maintained under a lower food supply were significantly smaller (height, width), lighter and contained fewer and smaller eggs than egg capsules laid by well-fed whelks.

Cheung & Lam (1999) similarly demonstrated that female *Nassarius festivus*, which were fed every 14 days, produced fewer and smaller egg capsules with fewer eggs per capsule, compared with females that were fed every 2 or 7 days. However, mean egg size (133-140 um) was similar under all feeding treatments. They also indicated that the egg capsules produced by whelks in the low-feeding treatment were more loosely packed with eggs, and their calorific value (including the eggs) was significantly lower. The low-ration group allocated only 1.4% of the consumed energy for reproduction, whilst the high-ration group was able to allocate 7.2% for reproduction (Cheung & Lam, 1999). When *N. festivus* was held under conditions of reduced food availability, more energy was invested in metabolic maintenance with less energy available for reproduction so that these gastropods increased their chances of survival until the next breeding period (Cheung & Lam, 1999).

A different approach to reduced food availability has been reported for other nassariid species such as *Nassarius pauperatus*. McKillup & Butler (1979) demonstrated that the number of egg capsules produced per female under a low feeding treatment was significantly higher than the number of capsules produced by females provided with a high level of food. Although the size of capsules was not significantly different between the treatments, the diameter of the eggs produced by whelks given the lower food ration was significantly larger and the number of eggs per capsule was significantly lower (McKillup & Butler, 1979). They suggested that when food availability is low the probability of the adults surviving to the next breeding season will be lower, thus an increased investment in reproductive output through the production of more egg capsules per female would be a suitable strategy (McKillup & Butler, 1979).

During the present study, *Nassarius reticulatus* responded to nutritional stress by reducing its reproductive output. This strategy may be an adaptive mechanism for scavengers that often face patchy and unpredictable food availability. Under a limited food supply more energy would be invested in maintenance and survival until the next breeding season, and consequently less energy would be available for egg production. The reduction of energy allocated to egg production is adaptive for scavengers since they inevitably endure periods of starvation (Cheung & Lam, 1999). Chester (1996) demonstrated that *Tenellia adspersa* reduced the size of the eggs when less energy was available, and thus were able to produce more eggs than they could have produced if the size of the eggs was kept the same.

Regularly fed *Nassarius reticulatus* produced egg capsules under laboratory conditions throughout most of the year. Spawning frequency increased as the seawater temperature increased during the early spring and summer, and the highest egg capsule production was recorded during June. Reproductive activity gradually decreased in July and August and an interruption of spawning was observed from September to October. Egg capsule deposition resumed after November and whelks were once again observed laying egg capsules during the winter, when seawater temperatures were as low as 7°C (January-February 2005). Breeding of *N. reticulatus* throughout Europe mainly takes place during spring and summer, usually between March and August (Rasmussen, 1973; Barnett *et al.*, 1980; Fretter & Graham, 1984; Barroso & Moreira, 1998). However, Lebour (1931) recorded that egg capsules are produced throughout the year in Plymouth (UK) except October and November. Winter reproduction has also been reported for other nassariid species such as *N. obsoletus* (Sastry, 1971) and *N. trivittatus* (Pechenik, 1978b). Tallmark (1980) reported that less egg capsules are produced per female *N. reticulatus* at lower winter temperatures. The average number of egg capsules produced per female *N. reticulatus* at 16°C was 33.5, whereas at lower temperatures of 12°C an average of 23 egg capsules per female was produced (Tallmark, 1980). Long spawning seasons may be the result of different age groups or individuals spawning at different times (Wigham, 1975). In species with a considerable range of bathymetric distributions the critical temperature for spawning may occur at different times of the year at different depths (Thorson, 1946).

Seasonal variations in egg capsule size produced by regularly fed *Nassarius reticulatus* were observed between March and November 2004. Egg capsules that were deposited in the laboratory during the summer and early autumn months had a significantly smaller height, width and weight than capsules deposited during early spring and winter. The diameter of eggs inside the capsules was significantly smaller during the summer, but there was, however, no difference in the number of eggs per capsule between the different seasons. Another example regarding seasonal variation in egg size is described by Pechenik (1978b). The egg capsules of *N. obsoletus* are morphologically very similar to those of *N. trivittatus* and both species initiate reproductive activity at similar temperatures (Scheltema & Scheltema, 1964; Pechenik, 1978b). However, veligers of *N. trivittatus* can emerge from the egg capsules at temperatures as low as 3.3°C (Pechenik, 1978b), whilst embryos of *N. obsoletus* do not develop in temperatures below 11-13°C (Scheltema, 1967). The number of eggs per capsule produced by *N. trivittatus* during winter was similar to the number of eggs per capsule produced by *N. obsoletus* during summer (Pechenik, 1978b). However, the size of *N. trivittatus* eggs laid during winter was larger than the size of *N. obsoletus* eggs laid during summer (Pechenik, 1978b).

In this study fecundity of *Nassarius reticulatus* increased during summer when seawater temperature and food availability were higher than the winter. Although more egg capsules were produced per female during summer, the size of the egg capsules and eggs was smaller than those recorded during winter. In order to sustain a constant number of eggs per capsule inside the smaller capsules, the eggs contained in the capsules had a smaller diameter. Due to the increased level of egg capsule production in summer it is likely that a smaller amount of energy was available for the construction of each egg capsule, thus smaller capsules and smaller eggs were produced. As Roff (1992) indicated, females with a certain amount of resources devoted to reproduction, produce more but smaller eggs in favourable environments, and few but larger eggs in unfavourable environments. Assuming that organisms have a finite and limited amount of energy available for reproduction, then they will only be able to increase the production of eggs if they produce smaller eggs (see reviews by Vance, 1973; Smith & Fretwell, 1974; Stearns, 1976; Bridges, 1993).

Larger eggs of *Nassarius reticulatus* produced during winter released larger veligers (see chapter 6). Spight (1976) demonstrated that larger eggs contained more yolk and hatch into larger larvae, which generally survive better than smaller ones, especially under non-favourable winter conditions. Development of larger eggs is slower, partly because yolk retards cleavage (Hughes, 1986). In order to reduce predation risks associated with prolonged development, capsules enclosing larger eggs are usually stronger than those containing smaller eggs (Hughes, 1986). By contrast, a reduction in the amount of capsular material (i.e. smaller and lighter weight capsules) during the summer may promote faster erosion of the capsule, thus allowing larvae to hatch at a smaller size (Chester, 1996). Indeed, as it is reported in the following section of this discussion, the duration of embryonic development is shorter during summer when the seawater temperature is higher. Smaller eggs of *N. reticulatus* that were produced during the summer had a shorter period of embryonic development and released smaller larvae (see chapter 6). Switzer-Dunlap & Hadfield (1977) indicated that members of the Aplysiidae with smaller egg size have shorter embryonic periods and they produce veligers of smaller size at hatching. Development of veligers during summer is probably faster due to the higher phytoplankton concentrations and the increased temperatures. The smaller summer larvae are able to survive and to grow rapidly, thus they soon catch up and reach the size of the larger winter larvae (see chapter 6).

Hatching of larvae from egg capsules of *Nassarius reticulatus* laid in the laboratory occurred successfully over a range of seawater temperatures. Incubation at seawater temperatures of 20, 17.5, 15 and 13°C resulted in 100% hatching of egg capsules, whilst at lower temperatures of 11 and 8.5°C 95% of egg capsules hatched successfully. The range of temperatures tested during the experiment was within the range of ambient seawater temperatures recorded in the laboratory and in the field during the reproductive period of *N. reticulatus*. Egg capsules were produced in the laboratory during most months of the year, thus embryonic development and release of veligers can successfully occur over a wide range of summer and winter seawater temperatures. The duration of embryonic development increased as the seawater temperature declined. Hatching of egg capsules started at 8, 12, 19 and 13 days after spawning for incubation temperatures of 20, 17.5, 15 and 13°C respectively, whereas release of veligers under lower temperatures of 11 and 8°C started after 31 and 35 days respectively. Hatching of egg capsules under incubation at 20, 17.5, 15, 13, 11 and 8.5°C was completed 32, 20, 25, 36, 38 and 56 days after spawning respectively. The shortest embryonic developmental period was observed at seawater temperatures of 17.5 and 15°C, which were within the temperature range recorded in the laboratory tanks during the June peak of the reproductive period. The seawater temperature in Rhosneigr intertidal lagoon during the summer was within the same range (15-18°C during May-June). Although hatching of egg capsules at 20°C started sooner (after 8 days) compared with the other seawater temperatures, it was completed after a longer period of time (32 days) than hatching of capsules at 17.5 or 15°C (20 and 25 days respectively). Temperatures as high as 20°C did not occur in the laboratory or in the field during the peak reproductive period, but were recorded in the intertidal lagoon during July and August, when spawning activity had diminished.

Table 5.6 A comparison of the effects of seawater temperature (°C) on the duration of embryonic development of *Nassarius reticulatus* under laboratory conditions reported in the present study and by Tallmark (1980). The number of days after spawning and the respective percentage of hatched egg capsules is presented.

Tallmark (1980)	Present study	Tallmark (1980)	Present study
20°C	**20°C**	**16°C**	**17.5°C**
8 days: start	8 days: start	12 days: start	12 days: start
10 days: 20%	10 days: 20%	14 days: 10%	14 days: 45.5%
12 days: 70%	12 days: 70%	22 days: 100%	20 days: 100%
17 days: 90%	17 days: 80%		
	32 days: 100%		
12°C	**13°C**	**8°C**	**8.5°C**
16 days: start	13 days: start	38 days: start	35 days: start
17 days: 5%	17 days: 11.1%	42 days: 5%	38 days: 5%
27 days: 80%	27 days: 77.8%	Terminated	56 days: 95%
	36 days: 100%		

Pechenik (1978b) indicated that in *Nassarius trivittatus* the time from spawning to hatching increased as the temperature decreased. Egg capsules produced in summer (21°C) required 5-7 days to hatch, in October (11.2°C) capsules hatched after 27-28 days, in November (9.6°C) the larvae were released after 35-42 days, whilst in

December (7.4°C) capsules began hatching after 55 days (Scheltema & Scheltema, 1964; Pechenik, 1978b). The results of the present study are similar to those reported by Tallmark (1980) who also investigated the effect of temperature on the embryonic development of *N. reticulatus* under laboratory conditions (table 5.6). Tallmark (1980) demonstrated that the time from egg laying to egg hatching was positively correlated with temperature, but hatching frequency decreased in both high (24°C) and low (8°C) temperatures. During the present study, where the incubation was continued for longer, 95% of the capsules finally released larvae at 8.5°C after 56 days.

In the present chapter the spawning behaviour and the reproductive output of *Nassarius reticulatus* have been described. The egg capsules of *N. reticulatus* in the Rhosneigr intertidal lagoon were laid on the underside of the stipes of the red seaweed *Chondrus crispus*, *Furcellaria lumbricalis* and *Plumaria plumosa*. Most of the capsules were deposited on the leathery and narrow stipes of the highly abundant *C. crispus*. In the laboratory most of the egg capsules were laid under the slots of the tank lids, where oxygenation of embryos was higher, or on the walls of the tanks. Breeding size classes of *N. reticulatus* have a shell length of 21 mm or greater. Fecundity of whelks, described as egg capsules·female^{-1} and period of spawning, increased in the larger size classes, but less energy was devoted to growth. Larger whelks produced more and bigger egg capsules·female^{-1}, with more and bigger eggs·capsule^{-1}. Limitations of food availability reduced the number of egg capsules produced per female and the duration of the breeding period. Egg capsules produced from whelks under a limited food supply were smaller and contained fewer and smaller eggs. Deposition of egg capsules by regularly fed individuals was recorded in all months of the year under laboratory conditions, even at seawater temperatures as low as 7°C. Reproductive activity increased in spring and summer, with peak fecundity during June, and gradually decreased in early autumn. Although very few egg capsules were produced between September and October, spawning activity resumed again after November and continued throughout the winter. The egg capsules produced during the summer at warmer temperatures were smaller and contained smaller eggs than the ones produced during the winter, although the number of eggs per capsule was constant between different seasons. The duration of embryonic development was greater at lower temperatures, although the hatching frequency was still very high even at low temperatures of 8.5°C.

CHAPTER 6

Larval development of *Nassarius reticulatus*

6.1 INTRODUCTION

Most prosobranch gastropods produce young that hatch as planktonic larvae and after a period of time they become benthic and attain the habitat of the adult (Fretter & Graham, 1994). Planktotrophic larval development occurs after production of a relatively large number of small-sized eggs (Thorson, 1950). The larvae acquire the necessary energy for growth by feeding on particulate matter during planktonic life (Thorson, 1950). The characteristics of a veliger larva are a) a shell which occupies the entire dorsal surface, b) a velum which is the swimming and feeding organ of the larva and c) a projection behind the mouth which is the developing foot (Fretter & Graham, 1994). A detailed review regarding the advantages and disadvantages of the planktonic mode of larval development is presented by Pechenik (1999). In summary, the advantages of free-living planktonic larvae, which have a greater dispersal ability, include the avoidance of competition for resources with adults, temporary reduction of mortality by benthic predators, a decreased likelihood of inbreeding in the next generation, and an increased ability to withstand local extinction (Pechenik, 1999). The possible disadvantages include dispersal away from favourable habitats, mismatches between larval and juvenile physiological tolerances, greater susceptibility to environmental stresses and planktonic predation, and various costs associated with metamorphosis under the presence of specific chemical cues and the delay of metamorphosis in the absence of these cues (Pechenik, 1999).

Planktotrophic larvae are released after deposition of a large number of small eggs which can be produced with a relatively small investment of energy, since the egg size is smaller than those produced for lecithotrophic larvae (Vance, 1973). Species with planktonic larvae have a greater ability than lecithotrophic larvae to exploit spatial and temporal patchiness in the adult environment in order to find a suitable settling site, although the overall percentage of successful recruits is often relatively low (Vance, 1973). Planktonic development increases the range of larval dispersion and can be advantageous when the adults have a limited dispersal ability and only exploit scattered patches of habitat that vary temporarily and randomly in quality (Crisp, 1976). The disadvantages of planktonic development are that veligers depend on an adequate food supply in their environment for survival and they are subject to planktonic predation (Vance, 1973). However, Vance (1973) suggested that starvation is not a significant source of planktonic larval mortality, since even the very small larvae of some bivalve molluscs are able to survive in filtered seawater containing no particulate food for more than a week. Although limited levels of food availability may not affect directly the survival of larvae through starvation, they can affect the growth rates of planktonic larvae (Vance, 1973). Predation and the transfer of larvae away from suitable settling sites are the most significant sources of larval mortality (Vance, 1973). Hence, by determining the amount of time that larvae might spend in the plankton and be subject to planktonic predation, food availability indirectly affects the survival of planktonic larvae (Vance, 1973).

Nassarius reticulatus larvae are an important constituent of the inshore and offshore plankton, and represent a large proportion of the food of the larger plankton-eating animals (Lebour, 1931). Lebour (1937) indicated that *N. reticulatus* veligers were very common in the coastal plankton off Plymouth throughout the year but especially in spring and summer. During a later study (1940-1945) in the same area, Lebour (1947) recorded *N. reticulatus* larvae from March to October, with the largest numbers present from March to June. In a more recent study that took place in Plymouth from 1969 to 1970, Fretter & Shale (1973) reported that *N. reticulatus* veligers were present in the plankton from January to September. In their study Fretter & Shale (1973) analysed the vertical distribution of *N. reticulatus* larvae in the offshore plankton in the coastal waters of Plymouth and suggested that larvae of all ages could be found over a 5-40 m depth range. A predominance of recently hatched larvae (1 ½ whorls) and young larvae (<2 ½ whorls) was recorded at 5m depth (Fretter & Shale, 1973). Usually younger larvae concentrated towards the surface and older larvae that were approaching metamorphosis were concentrated at greater depths (Fretter & Shale, 1973). However, *N. reticulatus* veligers, which are strong swimmers, can remain at an optimum depth and are usually found at a depth range 5 to 10 m, suggesting that when larvae are ready to settle they sink rapidly (Fretter & Shale, 1973).

The larvae of *Nassarius reticulatus* have a long free-swimming period lasting between 2 and 3 months and grow to a large size before losing their velum (Lebour, 1931; Fretter & Graham, 1984). According to Tallmark (1980) the planktonic phase of *N. reticulatus* from hatching to settlement required 25 to 32 days under laboratory conditions (20°C and 16°C respectively). The larvae of *N. reticulatus* have been successfully reared until the crawling stage in the laboratory on a diet consisting of *Nitzschia sp.* during the first stages and later enriched with *Skeletonema costatum* and *Thalassiosira gravida* (Lebour, 1931). Successful rearing of *Nassarius obsoletus* and *N. vibex* larvae until the stage of metamorphosis, using a diet of *Phaeodactylum tricornutum* and *Nitzschia closterium*, has been reported by Scheltema (1962b), who also managed to maintain juveniles up to the age of one year in the laboratory.

A detailed description of the larval stages of *Nassarius reticulatus* has been presented by Lebour (1931). The veligers of *N. reticulatus* hatch with a well formed smooth and transparent shell consisting of one whorl (Lebour, 1931). The width of the newly hatched larvae is about 250-350 um from the body whorl to the outer lip (Fretter & Graham, 1984). The outer lip of the shell is slightly drawn at the centre, curving inwards and forming two hollows to support the velum (Lebour, 1931). The projection grows into a conspicuous tooth and the hollows deepen as the velum grows (Lebour, 1931). The newly hatched veligers have a bilobed velum, 0.29-0.32 mm wide, with a thick ciliated rim round the margin and a smaller ciliated ridge on the underside (Lebour, 1931). These two ridges form a groove in which the food particles are collected and brought into the ciliated mouth (Lebour, 1931). The combined movements of the cilia on the velum and mouth ensure a constant supply of food to the animal (Lebour, 1931).

Planktotrophic prosobranch veligers feed on nanoplankton and microplanktonic cells, especially dinoflagellates and to lesser extent diatoms (Fretter & Graham, 1994). However, some larvae are capable of absorbing dissolved organic matter from seawater (Fretter & Graham, 1994). The veliger feeds continuously, by rising through the water with the velum fully extended and collecting food particles, then withdrawing the velum and sinking in order to rise again (Fretter, 1984). The beat of the velum cilia is outward over the velar edge, driving water posteriorly, and thus forcing the larva forwards and creating a strong water current with food particles towards the mouth (Fretter, 1967). The velum traps all the organic and inorganic particles of the correct size and selection of particles to some extent takes place when food is transferred from the velar cilia to the food groove and to the mouth (Webber, 1977). Unsuitable particles are entangled in mucus and left behind as the animal swims away (Lebour, 1931). When the stomach is full feeding stops so that digestion can proceed (Webber, 1977).

Nassarius reticulatus larvae have a border of brown pigment round the edge of the velum from the start of their planktonic life, while pigmentation on the velum of *N.*

incrassatus larvae appears later (Lebour, 1931; Fretter & Graham, 1984). Lebour (1931) reported the early presence of eyes on the base of very short tentacles and of statoliths at the base of the foot. Also the oesophagus, ciliated stomach, intestine, anus, liver, kidney and heart were all visible (Lebour, 1931). The foot is ciliated over its whole surface, has ventral colour pigmentation and it is always provided with an operculum (Lebour, 1931). After a few weeks the velum becomes 4-lobed, the shell begins to be spiral and the siphonal canal is formed (Lebour, 1931; Fretter & Graham, 1984). In *N. reticulatus* the velum remains slightly 4-lobed, while in *N. incrassatus* the lobes may grow to an enormous length (Lebour, 1931; Fretter & Graham, 1984). Larvae with a larger velum and longer lobes, such as *N. incrassatus*, remain in the plankton for longer and are usually found in offshore waters, while the larvae of *N. reticulatus* have a velum with smaller lobes and are usually found in more shallow water (Lebour, 1937).

When the larvae are close to metamorphosis the shell is 700-800 um wide and has 3 whorls (Lebour, 1931; Fretter & Graham, 1984) and the tentacles and siphon are long (Lebour, 1931). The front part of the foot grows into a squarish lobe which expands into two anterior horns and the animal is able to crawl (Lebour, 1931). An intermediate stage in which the larvae retain their velum, although the foot is already well developed, and the animal can swim or crawl at will, has been described for *Nassarius reticulatus* and *N. incrassatus* by Lebour (1931) and for *N. obsoletus* and *N. vibex* by Scheltema (1962b). The swimming/crawling stage precedes metamorphosis and has been named the "veliconcha" by Werner (1939). The precise moment of metamorphosis is determined by a correct combination of environmental and physiological conditions (Fretter, 1984). Metamorphosis involves a sequence of fast synchronised alterations. Soon the velum starts to dwindle gradually before disappearing completely (Lebour, 1931). Scheltema (1962b) reported that the velum of *N. obsoletus* and *N. vibex* was severed over 20-30 minutes and discarded. The tooth projection of the shell lip disappears and the shell edge is slightly crenulated (Lebour, 1931). The secretions from a gland, which lies ventral to the pedal ganglia and opens near the posterior tip of the mesopodium, are responsible for anchoring the veliger to its favourite settlement site at metamorphosis (Fretter & Graham, 1994). In *N. reticulatus* the gland is formed during the late larval stages and disappears after metamorphosis (Fretter & Graham, 1994).

Several environmental factors, such as direction and spectral quality of light (Thorson, 1964; Miller & Hadfield, 1986; Barile *et al.*, 1994; Johnson & Forward, 2003), temperature (Mann & Wolf, 1983), gravity (Crawford & Jackson, 2002), hydrostatic pressure (Cragg & Gruffydd, 1975; Cragg, 1980; Mann & Wolf, 1983), salinity (Campos & Mann, 1988) and tidal rhythmicity (Cronin, 1982), may elicit age-dependent behavioural responses and determine the distribution of marine invertebrate larvae. Usually they aggregate close to the water surface during their earlier developmental stages, while older larvae spend more time near to the bottom (Switzer-Dunlap & Hadfield, 1977; Cragg, 1980; Fretter, 1984; Kingsley-Smith *et al.*, 2005). The early larval stages are concentrated near the water surface where plankton is abundant (Cragg, 1980), and where current velocity is higher (Barile *et al.*, 1994), in order to achieve greater dispersal. When the larvae are close to metamorphosis they spend more time near to the seabed, so that the veligers have the opportunity to explore the substratum and increase their opportunities for settlement (Crisp, 1974; Hughes, 1986).

According to Thorson (1964), 82% of the species he studied had a positive phototaxis after hatching resulting in upward swimming, and a negative phototaxis near the time of metamorphosis, which resulted in their swimming near the bottom. Thorson (1964) indicated that geonegativity and photopositivity often combine to place early veligers in the surface waters even during the hours of darkness, in order to support further dispersal of the larvae. Barile *et al.* (1994) indicated that the larvae of *Strombus gigas* showed a strong photopositive phototaxis and a negative geotaxis during the early ontogenetic stages, although when the light intensity was high the larvae were photonegative. When veligers of *Nassarius reticulatus* hatch from their egg capsules they usually rise to the surface and aggregate in areas of high light intensity. However, when a phytoplankton bloom occurred, larvae avoided the surface water and aggregated in deeper water (Fretter & Shale, 1973; Fretter & Graham, 1994). Miller & Hadfield (1986) found that the larvae of the nudibranch *Phestilla sibogae* were highly photopositive during their early ontogenetic stages, and shifted to a photo-indifferent swimming behaviour as their age increased. Also the positive phototaxis of *Strombus gigas* larvae became reduced as they grew older (Barile *et al.*, 1994). As a population ages more larvae spend more time at greater depths and if the environment is favourable they metamorphose (Fretter & Shale, 1973).

In order to estimate dispersal potential and supply of larvae to local and distant settlement sites it is important to understand how variations in environmental conditions affect the length of larval life (Davis, 2000). The period of pelagic development for larvae of marine invertebrates is influenced by environmental factors such as food supply, temperature and salinity (Davis, 2000). The value of an algal species as food depends on the ability of larvae to ingest algal cells (size, shape), the accessibility of algal cells to the digestive system of larvae and the nutritional value of the algal cells (Pechenik & Fisher, 1979). Several studies have demonstrated that the quality and quantity of food provided to larvae affects their development. Lucas & Costlow (1979) indicated that larvae of *Crepidula fornicata* had a higher growth rate when fed with *Dunaliella tertiolecta* than when fed with *Pavlova lutheri*, and growth rate was greater as the concentration of the algae increased. Pechenik & Fisher (1979) demonstrated that the best growth of *Nassarius obsoletus* larvae was observed under a diet of *Thalassiosira pseudonana* and *Isochrysis galbana*, while the larvae did not grow measurably when provided with *D. tertiolecta*. Pechenik *et al.* (1996a) indicated that a rapid decline in phytoplankton concentration during the larval development of *C. fornicata* stimulated metamorphosis and reduced the larval dispersion period.

Veligers of *Nassarius obsoletus* can develop in salinities of 14‰ or higher (Scheltema, 1965). Scheltema (1965) observed that the growth rate of the larvae was higher as the salinity increased from 14‰ to 21‰, but for salinities above 21‰ there was no effect on growth. *N. obsoletus* larvae are able to withstand low salinity stress (15‰) at low seawater temperatures (Vernberg & Vernberg, 1975), and such conditions of low temperatures and low salinities are common during the spring when the larvae are released in the plankton (Vernberg & Vernberg, 1975). Growth of *Strombus gigas* larvae was reduced and mortality increased in high salinities (45 ppt) (Davis, 2000). Storm-induced salinity reductions at a level of 10‰ resulted in decreased growth rates of *Ilyanassa obsoleta* larvae (Richmond & Woodin, 1996).

Changes in environmental seawater temperature affect the length of larval life (Webber, 1977; Tallmark, 1980). Scheltema (1967) indicated that each degree drop in temperature, over a range from 28 to 20°C, increased the duration of *Nassarius obsoletus* larval life span by only 0.25 days, although when the seawater temperature was reduced from 20 to 16°C each degree drop in temperature added 2 days to the veliger's life. Larval development of *N. obsoletus* was suspended at 11.5°C, but started again when temperature was increased (Scheltema, 1967). The growth rate of *Crepidula fornicata* larvae was affected by environmental temperature, and changes in carbon content per larva reflected the effect of temperature on general metabolism and larval survival (Lucas & Costlow, 1979). High temperatures accelerated growth rate and reduced the number of days from hatching to metamorphosis for larvae of *Crepidula plana* (Lima & Pechenik, 1985) and *Strombus gigas* (Davis, 2000).

Termination of larval life involves two steps: settlement, which is reversible, and metamorphosis, which is an irreversible morphological alteration to the adult form (Crisp, 1974). Metamorphosis for a gastropod veliger involves the loss of the velum (Crisp, 1974) and occurs either as an increased sensitivity to some external inducing substances present in the seawater, the programmed eventual release of some endogenous stimulatory internal factor in the absence of external cues, or the degradation of some internal factor inhibiting metamorphosis (Pechenik *et al.*, 1996a). Although larvae may reach the stage where they are competent for metamorphosis they may delay their settlement and continue to grow until a favourable substratum is encountered (Scheltema, 1961; Fretter & Graham, 1994). Postponement of metamorphosis promotes settlement of individuals into suitable habitats most likely to support future survival and reproduction (Crisp, 1974). Scheltema (1961) indicated that *Ilyanasa obsoleta* might extend its planktonic life by 20 days after the swimming/crawling phase is attained if an appropriate substratum has not been found. However, metamorphosis cannot be delayed indefinitely and in most cases the larvae will finally metamorphose spontaneously after a maximum delay period, or they will die (Scheltema, 1961). Pechenik (1980) indicated that 94% of *Crepidula fornicata* larvae metamorphosed after a delay period of <30 days, 54% of *Bittium alternatum* larvae metamorphosed successfully after delaying their metamorphosis for up to 60-73 days, while only 17% of *I. obsoleta* larvae metamorphosed after a delay of 60 days. Species with very general adult habitat requirements produce larvae with limited delaying capabilities, while species with more patchily distributed adult niches are able to prolong larval development to a greater extent (Pechenik, 1980). Although many larvae will colonise new favourable habitats, some of them will never encounter a suitable place for settlement (Hughes, 1986).

A favourable substratum is a combination of physical, chemical and biological properties of the sediment (Scheltema, 1961; Fretter & Graham, 1994). According to Scheltema (1961; 1964) the physical characters of sediment, such as mean particle size and sorting, do not have a direct effect on metamorphosis. However, there are certain indirect effects of particle size on the biological properties of the substratum (e.g. the size of bacterial populations may be related to particle size). Pedersen & Page (2000) observed that *Polinices lewissi* larvae did not settle when autoclaved sediment was used and suggested that metamorphosis was probably induced by an organic factor in the sediment. The factor inducing metamorphosis appears to be a water-soluble substance that escapes from the sediment into the sediment-water boundary layer which may act either as an attractant or repellent (Fretter & Graham, 1994). Such factors might be related to the algal species on which the veligers settle (Aplysiidae species: Switzer-Dunlap & Hadfield, 1977), films of microrganisms including diatoms (*Littorina picta:* Struhsaker & Costlow, 1968), species used as food sources from adults (*Cerithiopsis tubercularis:* Fretter & Manly, 1977) or mucus trails from adults (Wells & Buckley, 1972). Switzer-Dunlap & Hadfield (1977) associated successful metamorphosis and optimum juvenile development of four species of Aplysiidae with specific algal species, and this was also supported by Stoner *et al.* (1996) working on *Strombus gigas*.

In some cases, however, less specialised physical factors appear to govern the settlement of larvae. McKillup *et al.* (1993) indicated that survival and settlement of *Nassarius pauperatus* larvae were higher in undrained sandflats, where the gradient of the sandflat was slight, the zone containing adults was wide, and extensive pools of water were formed during low tide. They suggested that a gently sloping sandflat may entrap larvae, and that the development and survival of larvae were greater in the water pools formed during low tide due to the elevated temperatures (McKillup *et al.*, 1993). Underwood (1972) suggested that settlement of the trochophore larvae of *Gibbula cineraria* on rocky shores was not stimulated by the presence of a suitable substratum, but instead the larvae were passively stranded in sheltered crevices as a result of wave and tidal action.

The aims of the present chapter were: 1) To describe the developmental stages and the swimming behaviour of *Nassarius reticulatus* veligers from hatching to metamorphosis, 2) To investigate the response of larvae to light and pressure stimuli, 3) To identify possible variations of these responses during the different ontogenetic stages, 4) To investigate possible seasonal variations regarding the size of the larvae at hatching, 5) To investigate the development, growth and survival of larvae under different ambient and constant temperatures, 6) To achieve the settlement and metamorphosis of larvae and rear the juveniles under laboratory conditions, 7) To investigate the effect of initial size at hatching on the growth rate of larvae until metamorphosis, and 8) To determine the effect of absence of sediment on larval metamorphosis.

6.2 MATERIALS AND METHODS

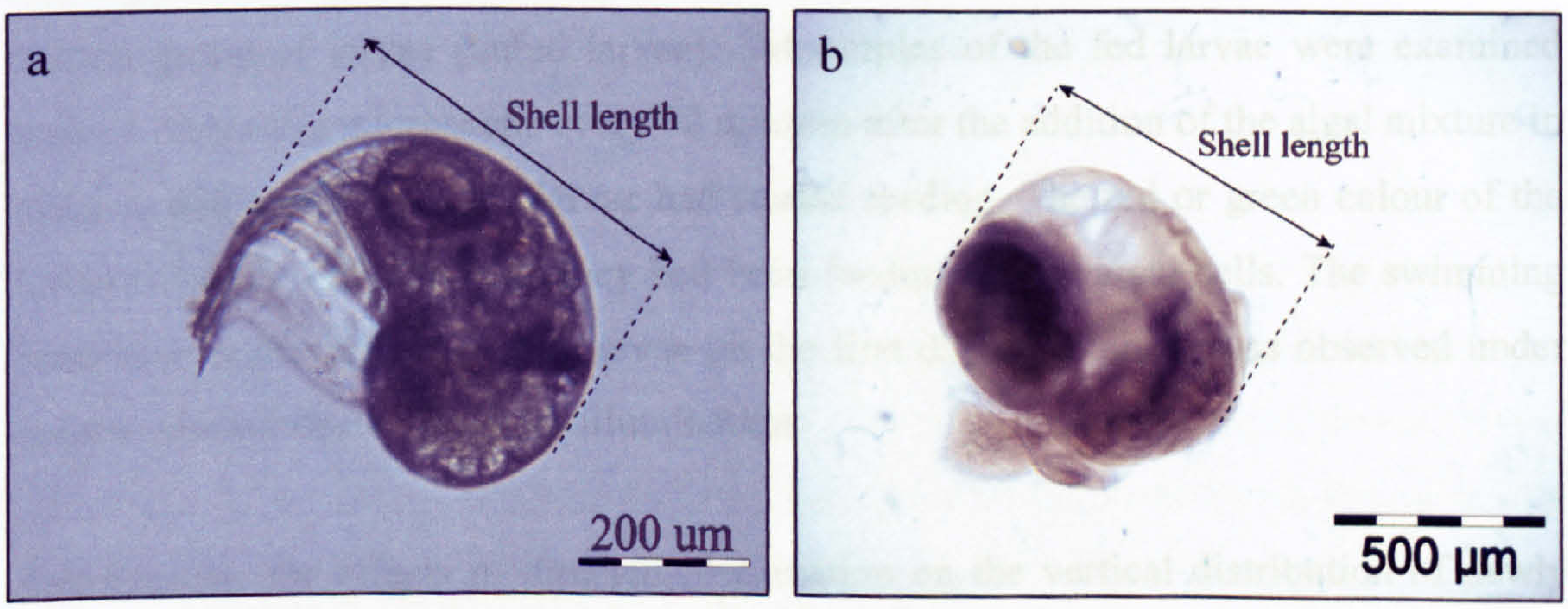
6.2.1 Egg hatching and hatching size of larvae

The egg capsules of *Nassarius reticulatus* that were laid under laboratory conditions at ambient seawater temperatures during the year (see chapter 5) were collected and placed in glass beakers with 1800 ml of 0.2 µm filtered ultra-violet irradiated seawater. The beakers were placed in a waterbath with running seawater at ambient temperature and covered with cling film in order to reduce evaporation. Constant aeration was provided in order to facilitate embryonic development. On the first day of hatching (1d old) a sub-sample of larvae was collected with a plastic pipette. The larvae were placed on a glass slide and excess water gently removed so that the larvae retracted into their shell thus facilitating their measurement. The shell length of the larvae was measured (figure 6.1) under a dissecting microscope equipped with an ocular calibrated micrometer. The seasonal variation in the size of 1d old larvae under seasonal ambient seawater temperatures was investigated.

6.2.2 Culture of larvae

On the first day of hatching the larvae were collected with a 63 um plankton mesh sieve and washed back into a 2 litre beaker with 1700 ml of 0.2 µm filtered ultra-violet irradiated seawater. The larval culture was provided with 100 ml of an algal culture mixture containing equal volumes (25 ml) of monocultures of *Rhinomonas reticulatus* (900 cells·μl^{-1}), *Pavlova lutheri* (3500 cells·μl^{-1}), *Tetraselmis chui* (100 cells·μl^{-1}) and *Skeletonema costatum* (2000 cells·μl^{-1}). Bayne (1965) and Bell (1987) reported that planktonic molluscs grow better on a mixture of phytoplankton species than they do on a single species. The seawater and algal culture mixture were renewed every three days. The temperature of the seawater used during renewals was acclimatised to the desired seawater temperature at which the larval culture was maintained, in order to avoid a sudden temperature shock to the larvae. The beakers containing the larvae were loosely covered with cling film in order to minimise evaporation. The larval cultures were provided with constant aeration, in order to enhance mixing of the algal culture, suspension of the larvae and to promote larval feeding.

Figure 6.1 Orientation of larvae during measurement of shell length under the microscope: a) an 11 day old larva with one shell whorl and b) a 31 day old larva with a second whorl formed. In both images the body of the larva is retracted into its shell.



Depending on the required seawater temperature the beakers containing the larvae were either placed on a water-bench provided with running seawater at ambient temperature, or placed in a temperature controlled waterbath. Seawater temperature was continuously monitored with a Tinytalk II temperature logger. Two weeks after hatching a thin layer of non-sterilised sand was supplied as sediment on the bottom of the beaker in order to promote larval settlement. After the third week of larval development a small amount of detritus, collected with a plastic pipette from the tanks where the adult gastropods were kept, was added onto the sediment. Also after the third week non-filtered seawater was used in an attempt to encourage the formation of a biofilm on the sediment.

Samples of 2-5 larvae at the various ontogenetic stages of development were collected and placed in a cavity slide with a small amount of seawater and covered with a glass cover slip. The different developmental larval stages were observed under a microscope (x4 & x10 objective) and photographed using a digital camera (ColorView-Soft Imaging System) attached to the microscope.

6.2.3 Response of larvae to illumination and pressure stimuli

Newly hatched (1d old) larvae were divided into 2 groups and placed in glass beakers with 0.2 μm filtered ultra-violet irradiated seawater. One of the groups (fed larvae) was provided with 100 ml of an algal mixture consisting of equal volumes (50 ml) of monocultures of *Rhinomonas reticulatus* (900 cells·μl^{-1}) and *Pavlova lutheri* (3500 cells·μl^{-1}). *Tetraselmis chuii* and *Skeletonema costatum*, the other two algal species usually added to the algal mixture, were not included because the size of their cells was too large to be digested by the newly hatched veligers. No algae were provided to the second group of larvae (unfed larvae). Subsamples of the fed larvae were examined under a dissecting microscope every 30 minutes after the addition of the algal mixture in order to determine when the larvae had started feeding. The red or green colour of the larval stomachs indicated that they had been feeding on the algal cells. The swimming behaviour of the fed and unfed larvae on the first day of hatching was observed under natural conditions of laboratory illumination.

Additionally, the effects of directed illumination on the vertical distribution of newly hatched fed and unfed larvae within the water column were investigated. Groups of 20 larvae (fed or unfed) were placed in 8 mls of seawater into a sealed (14 ml, 8 cm length) pressure syringe leaving 6 ml of air trapped between the water meniscus and the plunger. A narrow point cold light source was used as a photo-stimulus and directed either above or below the syringe. The experiments took place in a dark room so that no other sources of light could affect the behaviour of the larvae. The number of larvae aggregated above and below a mark halfway along the water body, after being illuminated for a 1-minute period, was counted. The distribution of the larvae was recorded in 5 replicate syringes with new larval samples.

The response of the larvae, following a combination of photo and pressure stimulation, was investigated. The vertical distribution before (standard atmospheric pressure) and after pressing the plunger (increased pressure) was examined in combination with illumination either above or below the syringe. Assessment of pressure increments induced was based on Boyle's law $P_1 \times V_1 = P_2 \times V_2$, where P_1 is the standard atmospheric pressure (1atm =101.325 kPa =1 bar), P_2 is the increased pressure after depressing the plunger, V_1 is the volume of the air bubble inside the tube before depressing the plunger (V_1=6ml) and V_2 is the volume of the air bubble after depressing the plunger. The number of larvae aggregated above and below the halfway mark along the water body was recorded as the pressure was gradually increased (1 ml volume reduction per minute) for a 5-minute period.

Following observations on the first day of hatching, the fed and unfed larvae were grouped together and maintained under a constant temperature of 20°C (details as in section 6.2.2). The swimming behaviour and response to illumination and pressure stimuli of not recently fed larvae (algal mix added the previous day) and recently fed larvae (algal mix added 1.5 hours before observations) was investigated for larvae at different developmental stages from hatching to metamorphosis.

6.2.4 Larval development under ambient temperature

The increase in size of the larvae between hatching and settlement, which were released from egg capsules during different months under ambient temperature, was recorded. The beakers containing the larval cultures were placed on a water-bench provided with running seawater at ambient temperature. The seawater temperature inside the larval culture vessels in practice was ~2°C higher in the summer and ~4°C higher during the winter than the actual ambient seawater temperature. A sample of 10 larvae was collected every 3 days using a plastic pipette, the larvae placed on a glass slide, excess water removed so that the larvae would retract into their shell, and their shell length measured under a dissecting microscope equipped with a calibrated ocular micrometer. Sampling of larvae and measurement of their size continued until metamorphosis and settlement.

6.2.5 Larval development under constant temperature

The increase in size of the larvae, between hatching and settlement, was recorded under conditions of constant temperature (20, 17.5, 15.5, 13, 12, 11 and 8.6°C). The beakers containing the larval cultures were placed in a controlled-temperature waterbath. The larvae held at each constant temperature were hatched at different times of the year and were held at a constant temperature similar to that of the ambient seawater temperature at the time of hatching. This was carried out to avoid the effect of abrupt temperature changes on larval development. A sample of 10 larvae was measured every 3 days as described above.

6.2.6 Larval survival

Two larval cultures were maintained under constant temperatures of 17.5 and 20°C, which were within the temperature range that induced successful settlement (see results section). The number of living larvae$\cdot$ml^{-1} of culture was estimated on the first day after hatching (1d) and measured every 3 days until metamorphosis. The larvae were distributed evenly throughout the water column using the vertical movement of a plunger. The plunger consisted of a plastic rod with a perforated perspex disk attached to its end. Six 1 ml samples were collected using a pipette and placed in a watch-glass. The number of swimming larvae in each 1 ml sample was counted under a dissecting microscope and the number of larvae in the total volume estimated. The percentage survival of larvae was determined from the first day after hatching until metamorphosis of the larvae.

6.2.7 Juvenile development

Metamorphosis and settlement of *Nassarius reticulatus* larvae were induced under ambient or constant seawater temperatures only within the range 16 to 20°C (see results). The larvae were competent for metamorphosis only when they started losing their velum and developing their foot. At this stage the larvae could still swim or crawl at will. When the larvae approached metamorphosis the larval cultures were checked daily for fully metamorphosed individuals. The overlaying water of the culture beaker was carefully decanted in order to leave a small volume of seawater to wash the retained sediment into a white plastic observation tray to a depth of 1-2 mm. The tray was left to stand for 10 minutes and the successfully metamorphosed larvae were located from the trail marks that appeared as they actively crawled across the sediment. The trail marks were easily visible against the white colour of the tray when the sediment was thinly spread. Crawling individuals were collected with a plastic pipette and transferred into a small plastic tank (L:17 x H:10 x D:11cm) containing a 5-10 mm layer of sand and thin gravel sediment. The top of the tank was covered with nylon mesh net in order to prevent the escape of the small juveniles through the slots of the tank lid. The tanks were provided with running seawater at ambient temperature. The newly metamorphosed juveniles were provided with small pieces of fish or mussel flesh several times a week in order to investigate when their scavenging mode of life was initiated. The larvae hatched at ambient seawater temperature between June and July 2003 successfully metamorphosed 41 days after hatching and their development as juveniles was followed until the age of 500 days, when the animals were sacrificed for statolith extraction (see chapter 4). The shell length of juveniles was regularly measured (usually monthly) under a dissecting microscope with a calibrated ocular micrometer and their growth rate was estimated.

6.2.8 Comparison of larval development of "small" and "big" larvae

Having established that the size of larvae at hatching depended on seawater temperature (see results), a comparison between the development of "big" and "small" larvae was performed. Small larvae hatched during September with an initial mean shell length of 291.1 um, whereas the bigger larvae hatched in May with an initial mean size of 340 um. All the larvae were reared under the same optimum temperature of 17.5°C. A sample of 10 larvae was collected every 3 days with a plastic pipette and shell length measured under a dissecting microscope equipped with a calibrated ocular micrometer.

6.2.9 Comparison of larval development with and without the addition of sediment

In all the larval cultures described above a thin layer of non-sterilised sand was added in the beaker two weeks after the larvae were hatched, and the sediment was enriched with detritus the third week after hatching to promote settlement and metamorphosis (see section 6.2.2). In this experiment larval development and competency for metamorphosis without the addition of sediment were examined. Two larval cultures were established at 17.5°C using larvae hatched during September (initial mean size of 312.5 um and 320 um respectively). This time no sediment was added in the beakers after the second week from hatching as was usually done. A sample of 10 larvae was collected every 3 days using a plastic pipette and their shell lengths measured under a dissecting microscope equipped with a calibrated ocular micrometer. The development and growth rate of the larvae incubated without sediment was compared with larvae developed under the same temperature with a supply of sediment ("small" and "big" larvae as referred to in section 6.2.8).

6.3 RESULTS

6.3.1 Larval developmental stages

The developmental stages of *Nassarius reticulatus* larvae are presented in figure 6.2. The newly hatched larvae have a smooth transparent shell consisting of one whorl. The eyes and statoliths are clearly visibly from the very early developmental stages. The outer lip of the shell curves inwards so that two hollows are formed in order to support the velum. This projection of the shell forms a characteristic tooth. The velum is bilobed and ciliated with a continuous brownish border along the inside of the margin. On the second day after hatching the average width of the velum was 0.36 mm. The veligers collect food particles while swimming with the help of the velum. The stomach is also ciliated and food material is continuously moving inside it. During the first week after hatching the foot is not yet well developed but colour pigmentation on its ventral surface is visible. When the veligers are about 19 days old an indentation at each side of the velum indicates the first signs of a 4-lobed velum. The width of the velum grows larger. In 31-day old veligers the velum becomes 4-lobed and the animal now moves by flapping the lobes. The foot is perceptibly longer and the shell consists of more than one whorl. When the veligers are about 35 days old the foot has grown out into a square form in front and the colour pigmentation is stronger. The anterior lobe of the foot expands into two horns and the animal is able to crawl. At this stage the veliger can swim or crawl at will (swimming/crawling stage). The outer shell tooth and the velum start to diminish. When the foot and the radula develop completely the planktotrophic phase is abandoned and the veligers metamorphose. At the stage of metamorphosis the shell consists of 2-3 whorls and the tentacles and siphon are well formed.

6.3.2 Swimming behaviour of larvae

Recently hatched unfed larvae spent most of their time near the upper surface of the water. When algal culture was provided to the larvae they started swimming actively, usually moving in a vertical direction downwards or upwards, or spinning around themselves. The stomach of newly hatched larvae, which were provided with algal mixture, had a bright red and green colour just one hour after hatching, indicating that

Figure 6.2 Developmental stages of *Nassarius reticulatus* larvae: a) Recently hatched veliger (1-day old) swimming with its bilobed velum expanded. The continous brownish border on the inside of the velum margin is clearly visible. b) Recently hatched veliger (1-day old) in which the statoliths can be seen through the transparent shell. c) Lateral view of a 4-day old veliger. The shell forms a characteristic tooth on its edge in order to support the velum. Colour pigmentation on the ventral surface of the foot is visible. d) 4-day old larva with its growing velum fully expanded. e) The size of veligers is rapidly increasing (7-day old). f) View from above of a 7-day old larva showing details (cilia) of the bilobed velum. e: eyes, f: foot, st: statoliths, th: shell tooth, v: velum. (continues in next page)

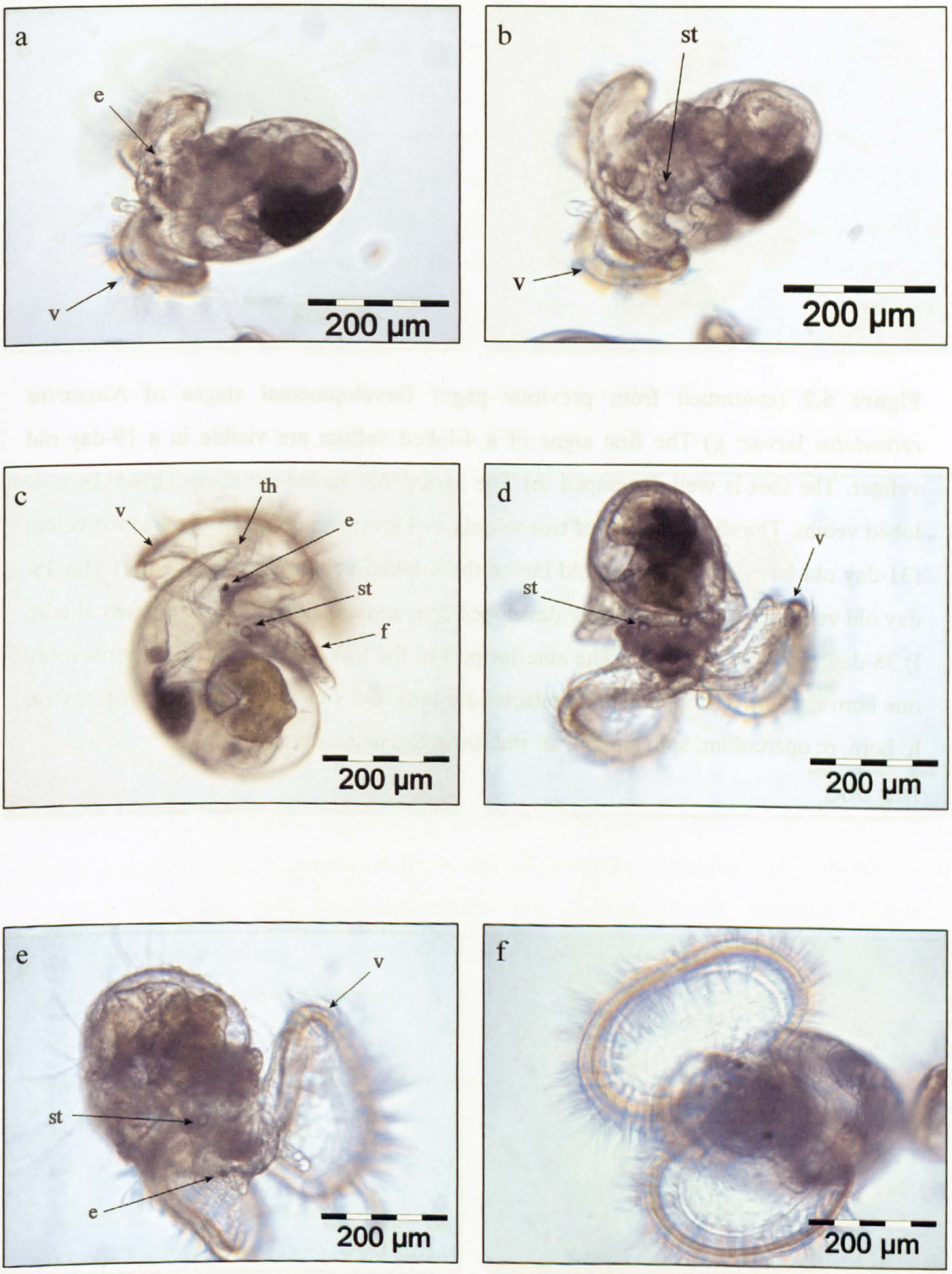

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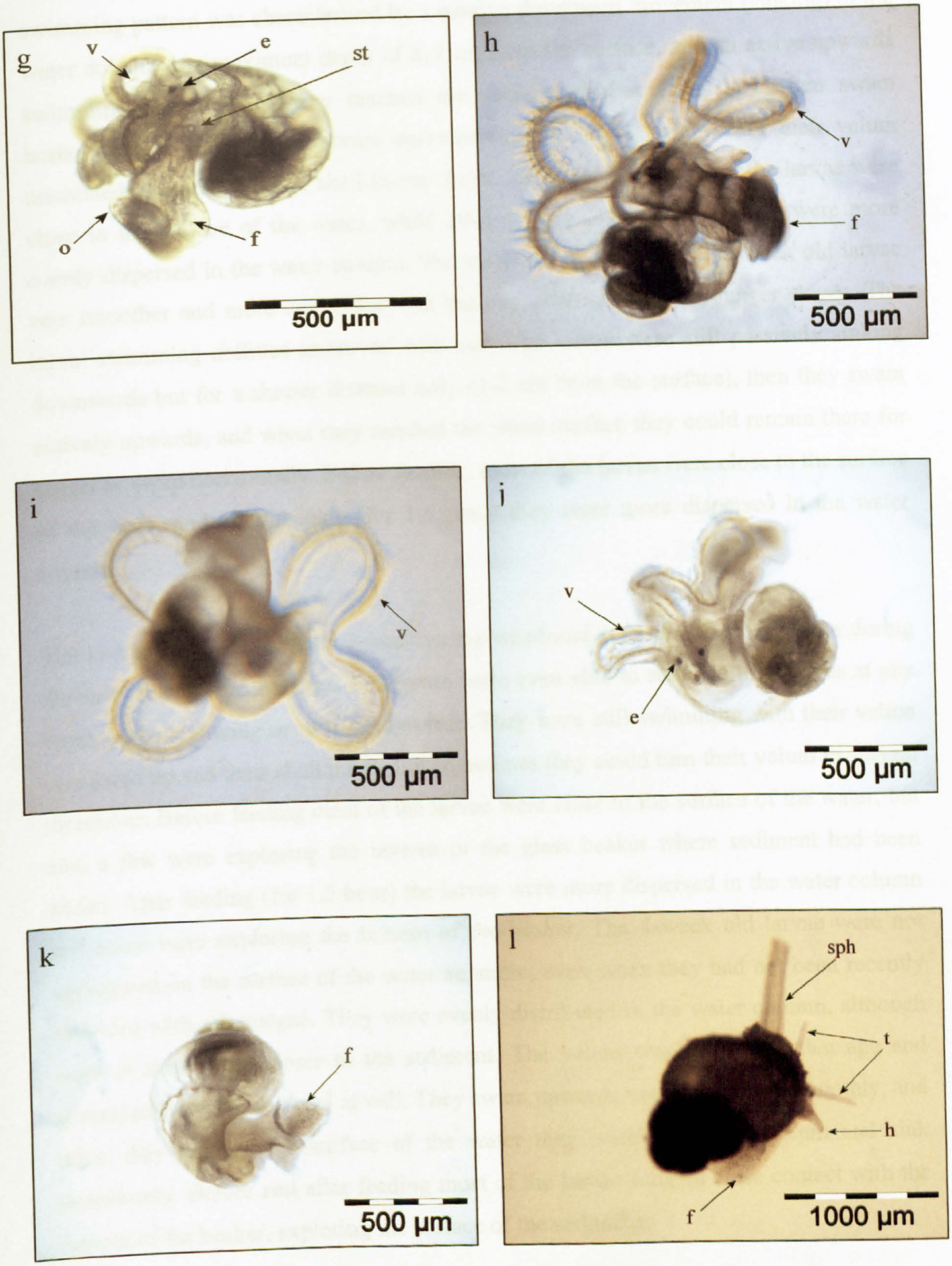
Figure 6.2 (continued from previous page) Developmental stages of *Nassarius reticulatus* larvae: g) The first signs of a 4-lobed vellum are visible in a 19-day old veliger. The foot is well developed. h) The 31-day old larvae are characterised by a 4-lobed velum. The shell consists of two whorls. i) View from above of the 4-lobed velum (31-day old larva). j) In 35-day old larvae the 4-lobed velum is diminished. k) The 35-day old veligers have a completely developed foot and are able to swim or crawl at will. l) 38-day old settled juvenile. The anterior part of the foot expands into two horns (only one horn shown). The siphon and tentacles are long and well developed. e: eyes, f: foot, h: horn, o: operculum, sph: siphon, st: statoliths, t: tetancles, v: velum.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they are able to feed as soon as they hatched. When the larvae were 1-week old their swimming pattern was characterised by a passive downward movement (sinking) in the water column to a maximum depth of 6-7 cm from the surface, and an active upwards swimming phase. When they reached the surface of the water they often swam horizontally with brief and abrupt movements. The larvae swam with their velum orientated upwards and their shell facing down. Before feeding most of the larvae were close to the surface of the water, while after feeding (for 1.5 hour) they were more evenly dispersed in the water column. The swimming pattern of the 2-week old larvae was smoother and more controlled than the one observed in the younger stages. The larval swimming abilities improved with age. The larvae were still passively sinking downwards but for a shorter distance only (1-2 cm from the surface), then they swam actively upwards, and when they reached the water surface they could remain there for longer or swim horizontally. Before feeding, most of the larvae were close to the surface of the water and after feeding (for 1.5 hour) they were more dispersed in the water column.

The swimming pattern of the 3-week larvae was more smooth and slower than during the early development stages. The larvae were even able to stabilise themselves at any depth without sinking or moving upwards. They were still swimming with their velum orientated up and their shell down, but sometimes they could turn their velum in several directions. Before feeding most of the larvae were close to the surface of the water, but also a few were exploring the bottom of the glass beaker where sediment had been added. After feeding (for 1.5 hour) the larvae were more dispersed in the water column and some were exploring the bottom of the beaker. The 4-week old larvae were not aggregated on the surface of the water anymore, even when they had not been recently provided with microalgae. They were evenly distributed in the water column, although most of them were closer to the sediment. The velum was 4-lobed at that age and several could swim or crawl at will. They swam upwards very slowly and smoothly, and when they reached the surface of the water they could fold their velum and sink downwards. Before and after feeding most of the larvae were in close contact with the bottom of the beaker, exploring the surface of the sediment.

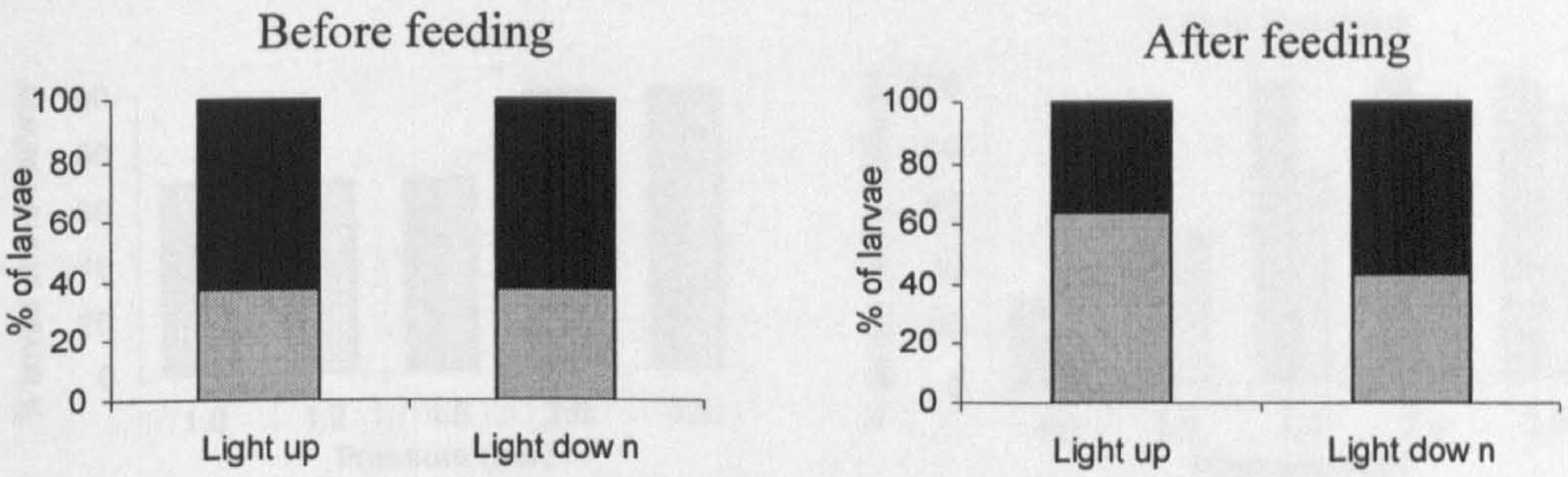
6.3.3 Response to illumination and pressure stimuli

The experiments investigating illumination and pressure stimulation were conducted under laboratory conditions using larvae of different developmental stages, before and after feeding. Initially the phototactic response of the larvae was examined under standard atmospheric pressure (figure 6.3), and then the effect of combined light and pressure stimuli on the vertical distribution of the larvae was investigated (figure 6.4). The distribution of recently hatched unfed larvae was not affected by the direction of the light. A higher percentage of larvae gathered near the water surface independently of the light direction. Their swimming behaviour was not clearly phototactic after they had been provided with microalgae. Although more larvae seemed to avoid light and gather in the lower water levels when they were illuminated from above, they were equally distributed in the water column when they were illuminated from below. One week old larvae still did not display obvious phototactic swimming behaviour. The non-fed larvae were equally distributed in the water column when they were illuminated from above. However, when they were illuminated from below more larvae were attracted by the light. After being fed, 1-week old larvae avoided the light source when illuminated from above, but were equally distributed when they were illuminated from below. During the first week of their life *Nassarius reticulatus* larvae were not strongly phototactic, however there were some indications that unfed larvae were photopositive and fed larvae were photonegative. Larvae 2 to 4 weeks old were clearly attracted by light before they were provided with algal culture and they displayed a strong photopositive swimming behaviour. After the larvae had been feeding for a 2-hour period they were not phototactic and they remained at the lower water levels, close to the bottom of the experimental beaker, independently from the light source direction.

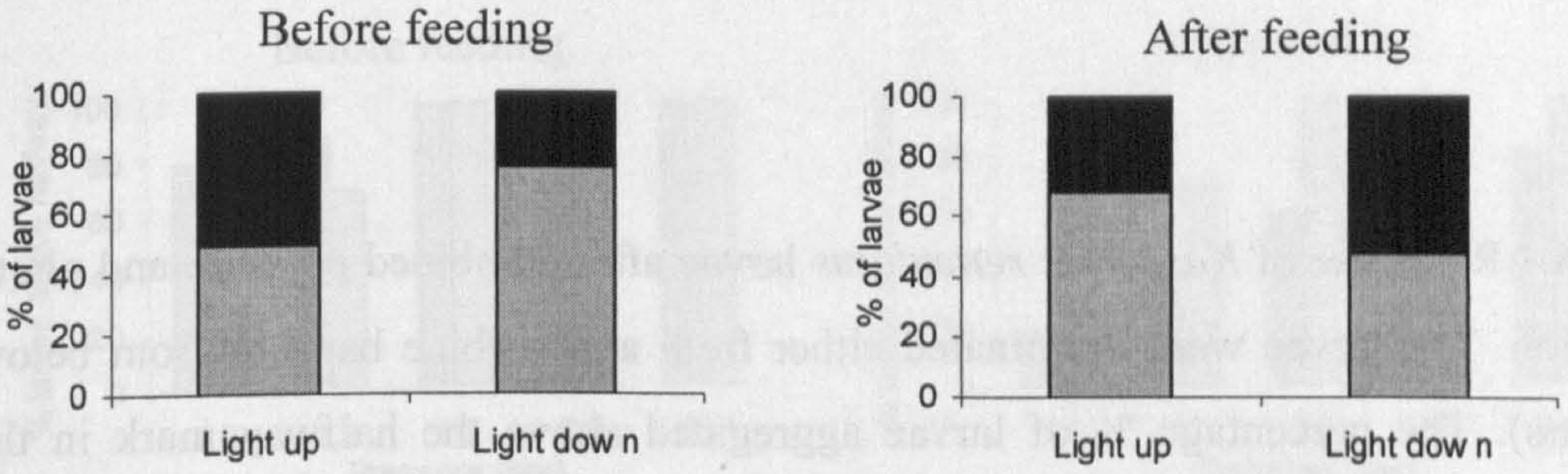
When the larvae experienced increased pressure they moved towards the water surface, thus indicating a strong barometric sensitivity. The same behaviour was observed in larvae of all ages, from the first day of hatching until the age of 4 weeks. Even during the developmental stages when a strong photopositive swimming behaviour was observed (non-fed larvae of 2-4 weeks old), all the larvae moved up towards the water surface when the pressure was increased, independently of the light source direction. However, older larvae were less sensitive to pressure increases. As has been previously indicated, fed larvae of 2-4 weeks old spend most of their time away from the surface of

Figure 6.3 Distribution of *Nassarius reticulatus* larvae in the water collumn during laboratory experiments with photo stimulation from different directions. The response of larvae of different ages before and after feeding was examined. The black areas represent % precentage of larvae aggregated at the upper water level, whilst the grey areas represent % precentage of larvae aggregated at the lower water level. The direction of the light source is indicated on the x-axis.

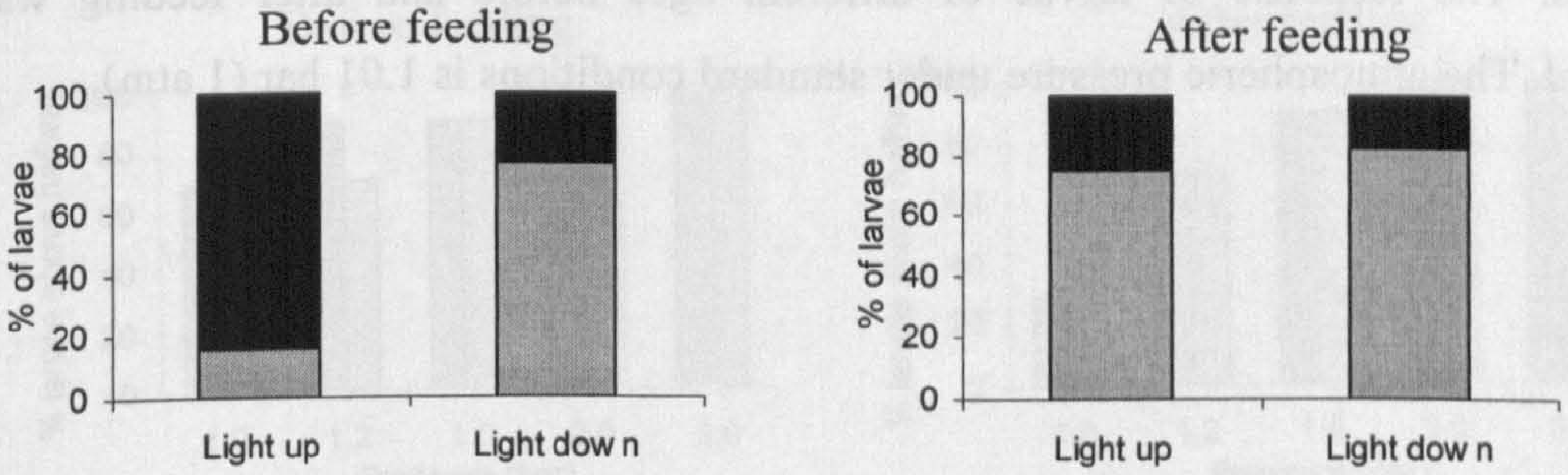
1-Day old



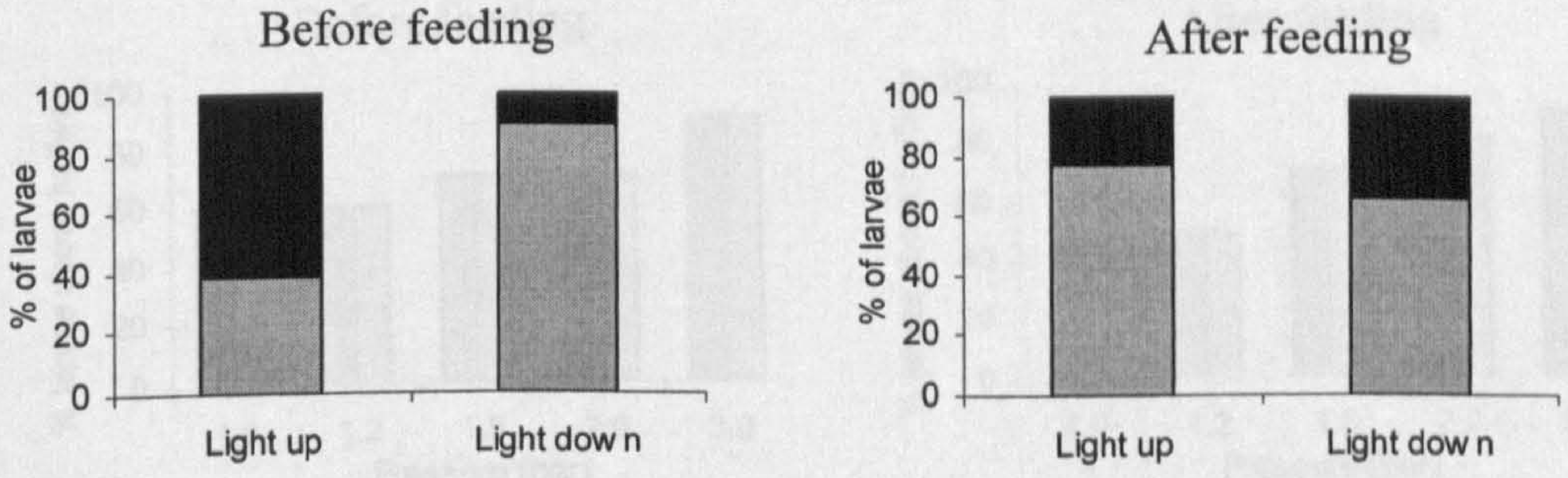
1-Week old



2-Week old



3-Week old



4-Week old

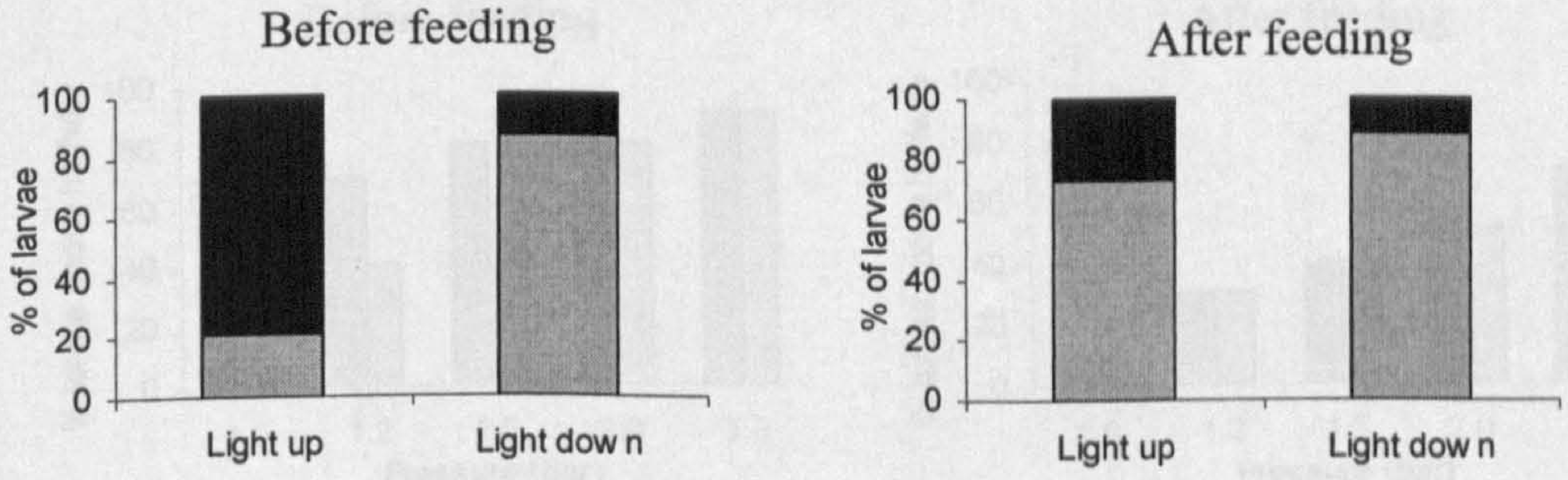
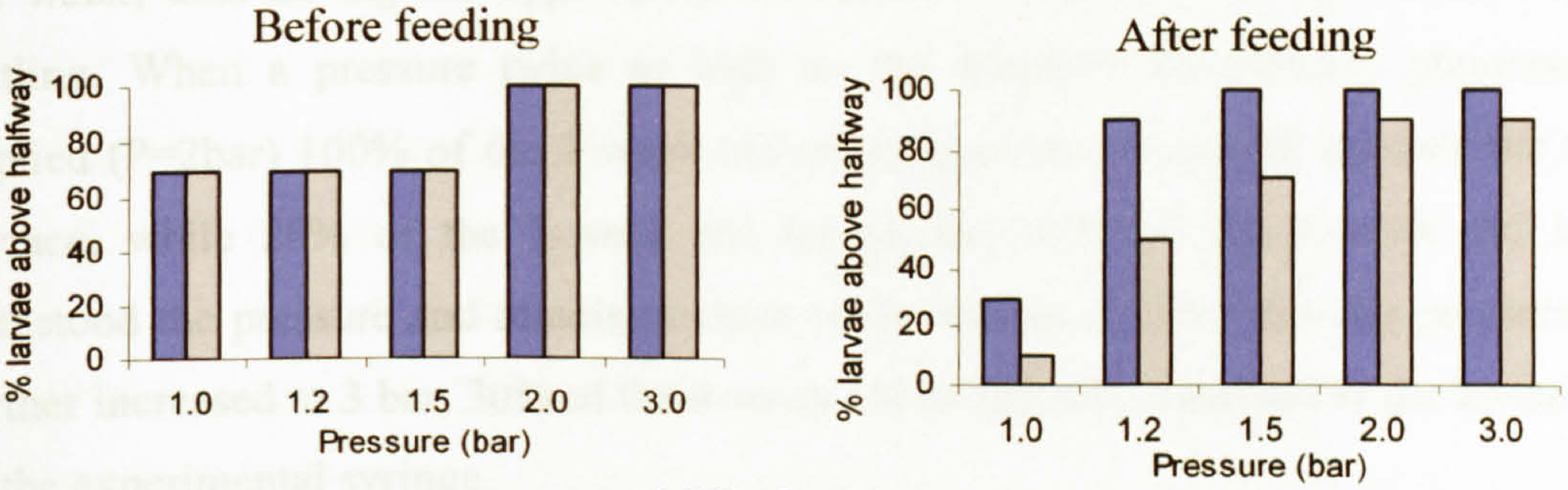
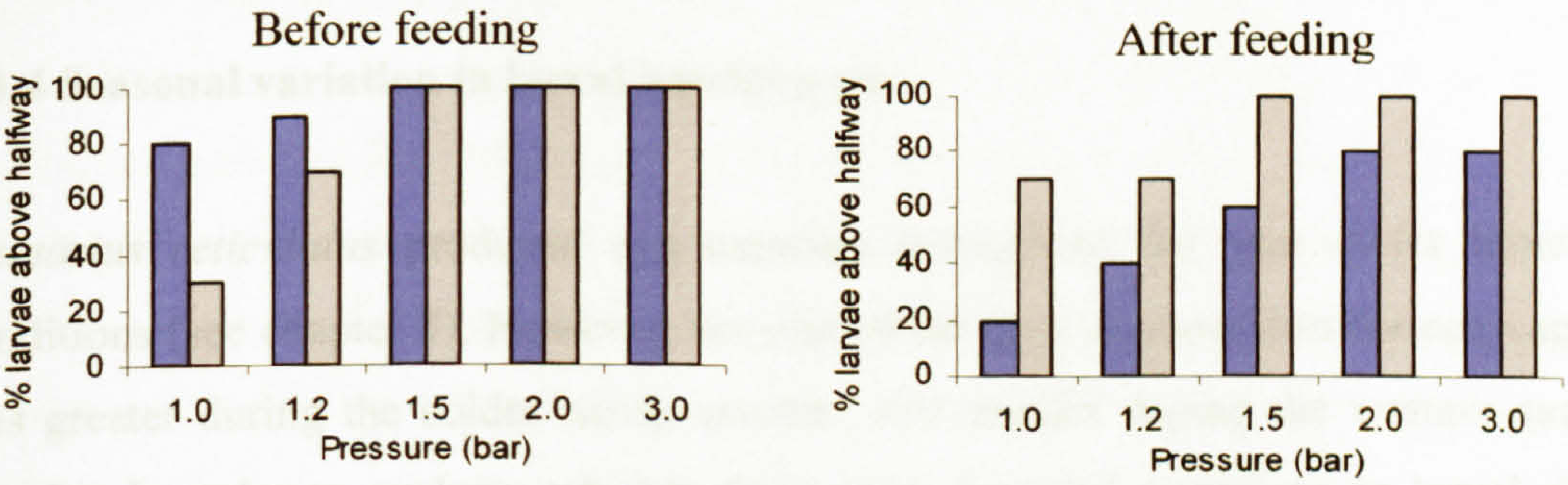


Figure 6.4 Response of *Nassarius reticulatus* larvae after combined pressure and photo stimulation. The larvae were illuminated either from above (blue bars) or from below (grey bars). The percentage % of larvae aggregated above the halfway mark in the experimental syringe (close to the water surface) was estimated for different air pressures. The response of larvae of different ages before and after feeding was examined. The atmospheric pressure under standard conditions is 1.01 bar (1 atm).

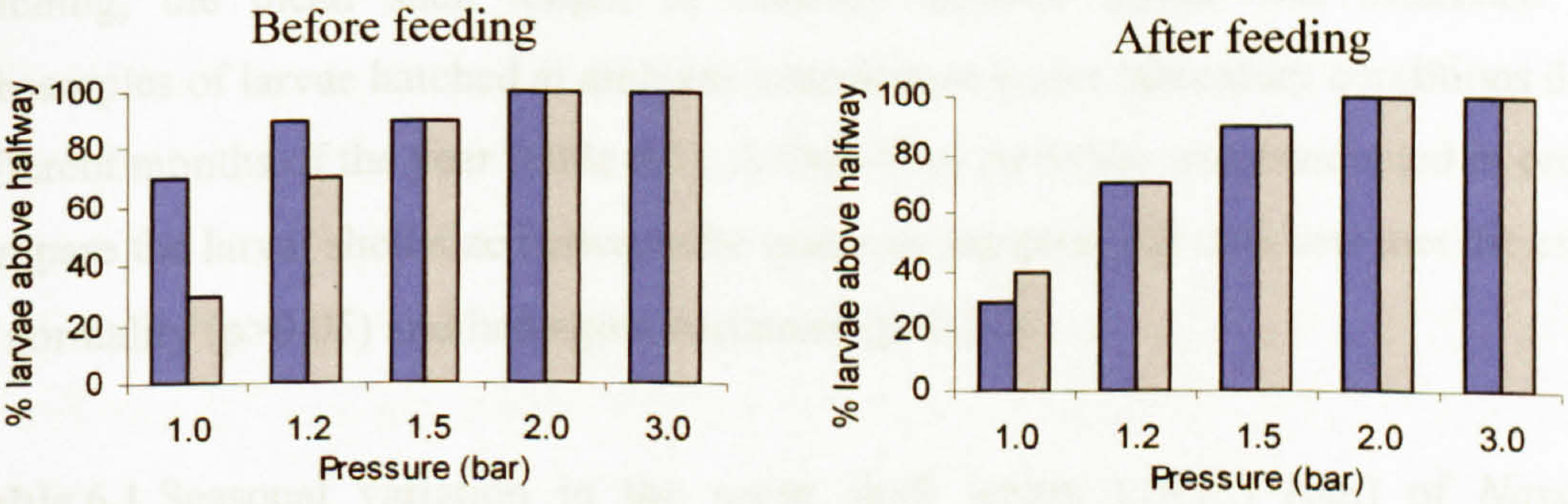
1-Day old



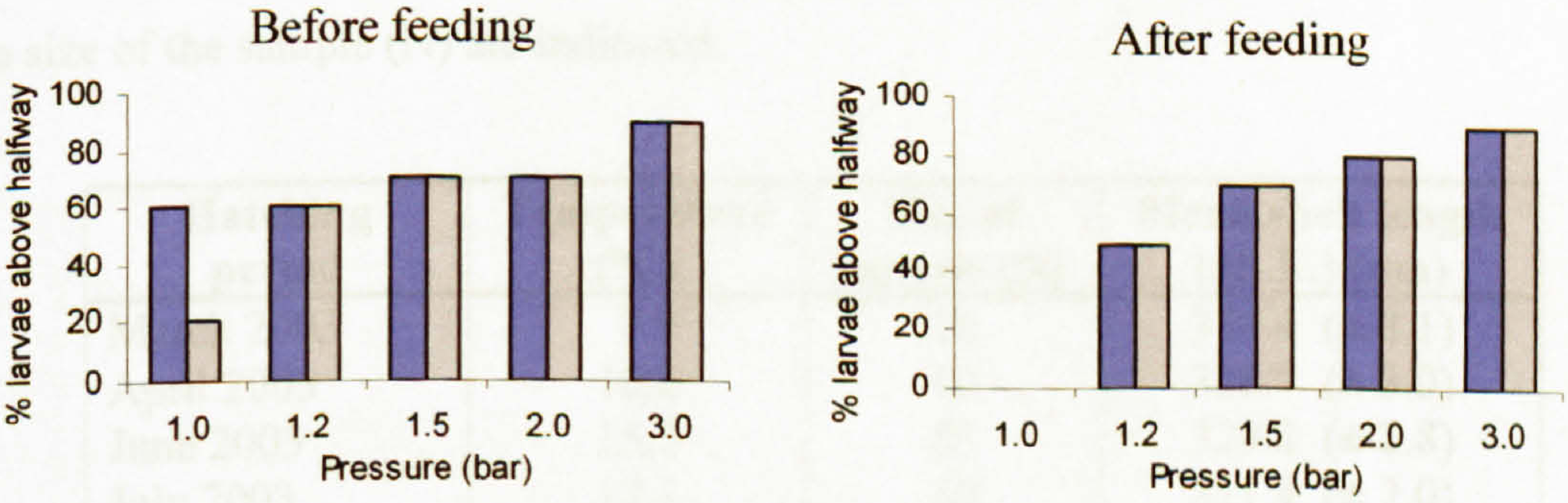
1-Week old



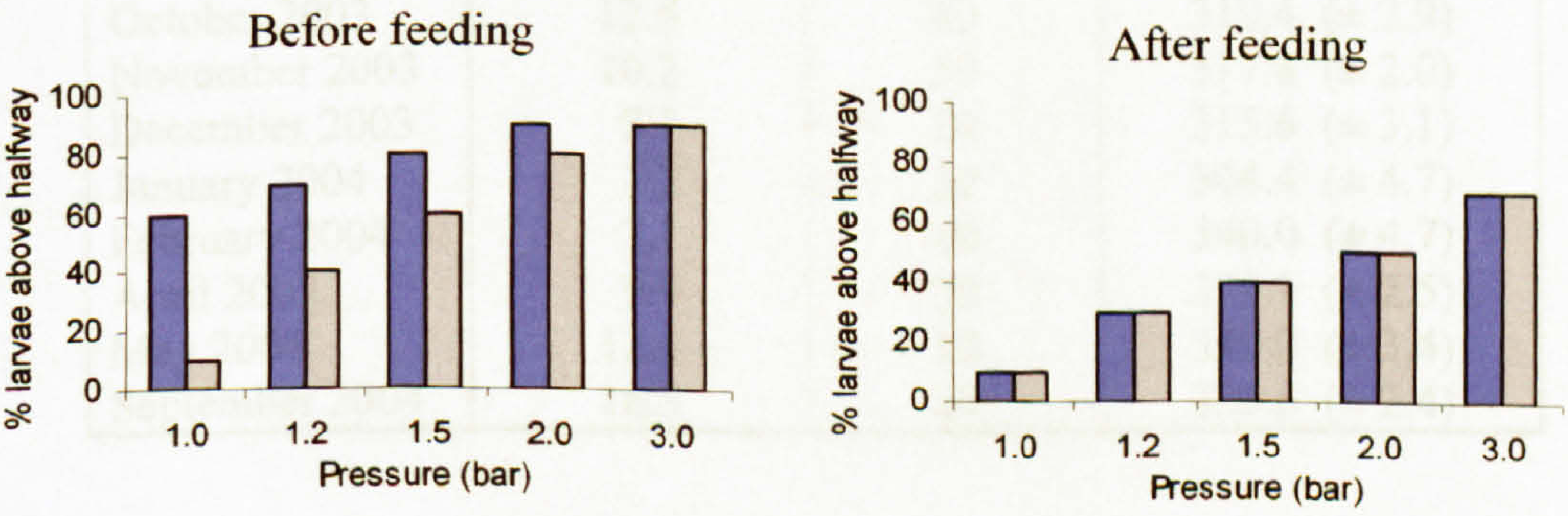
2-Week old



3-Week old



4-Week old



the water, thus having the opportunity to explore the bottom sediment before finally settling. When a pressure twice as high as the standard atmospheric pressure was applied (P=2bar) 100% of the 2-week old or younger larvae moved towards the water surface, while 20% of the 3-week old larvae and 50% of the 4-week old larvae withstood the pressure and remained close to the bottom. Even when the pressure was further increased to 3 bar, 30% of the 4-week old larvae still remained at the lower level of the experimental syringe.

6.3.4 Seasonal variation in larval hatching size

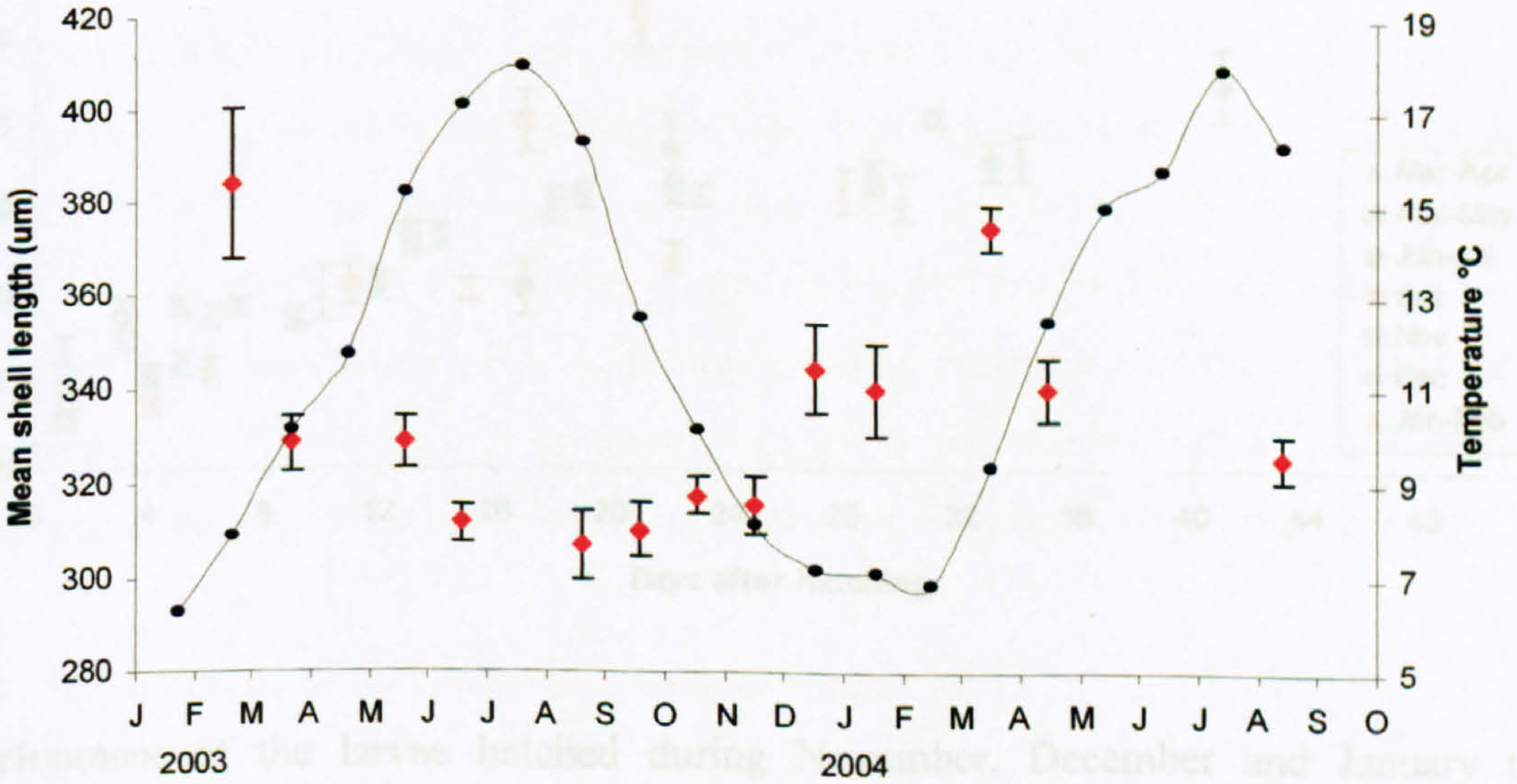
Nassarius reticulatus produced egg capsules throughout the year under laboratory conditions (see chapter 5). However, the size of the eggs contained in the egg capsules was greater during the colder winter months, and smaller during the warmer summer months. In order to evaluate whether there were seasonal variations in larval size at hatching, the mean shell length of recently hatched larvae was estimated from subsamples of larvae hatched at ambient temperature under laboratory conditions during different months of the year (table 6.1). A One-Way ANOVA was conducted in order to compare the larval shell size between the seasonal samples. All data sets met the criteria of normality (p>0.05) and had equal variances (p>0.05).

Table 6.1 Seasonal variation in the mean shell length (±S.E.) (um) of *Nassarius reticulatus* larvae on the first day of hatching. The mean temperature of each month and the size of the sample (N) are indicated.

Hatching period	Temperature (°C)	Size of sample (N)	Mean shell length (±S.E.) (um)
March 2003	7.9	10	384.4 (± 8.1)
April 2003	10.2	10	328.9 (± 3.0)
June 2003	15.2	65	329.2 (± 2.8)
July 2003	17.1	60	311.9 (± 2.0)
September 2003	16.3	30	307.4 (± 3.7)
October 2003	12.5	30	310.4 (± 2.9)
November 2003	10.2	50	317.8 (± 2.0)
December 2003	8.1	20	315.6 (± 3.1)
January 2004	7.2	20	344.4 (± 4.7)
February 2004	7.1	10	340.0 (± 4.7)
April 2003	9.4	50	375.1 (± 2.5)
May 2004	12.4	10	340.0 (± 3.4)
September 2004	16.3	20	325.6 (± 2.4)

There was a significant difference in larval shell length at hatching between the different months (F=52.36, $p<0.001$) (figure 6.5). Larvae hatched during March 2003 and April 2004 had the greatest shell length of 384.4 um and 375.1 um respectively, while the larvae hatched during the summer (July) and early autumn (September and October) of 2003 had the smallest shell lengths (311.8, 307.4 and 310.4 um respectively). Larvae that hatched about 1-2 months after egg laying (see chapter 5), during March 2003 and April 2004, were released from egg capsules produced during the coldest months of the year (February 2003: 6.3°C, March 2004: 6.9°C) and these larvae had the greatest size. Accordingly, the smallest larvae hatched during July, September and October 2003 were released from egg capsules that were produced during the warmest months (June 2003: 15.2°C, July 2003: 17.1°C, August 2003: 18.0°C, September 2003: 16.3°C). The production of smaller eggs during the warmer months resulted in the release of small larvae, whilst larger eggs produced during the colder months released the largest larvae.

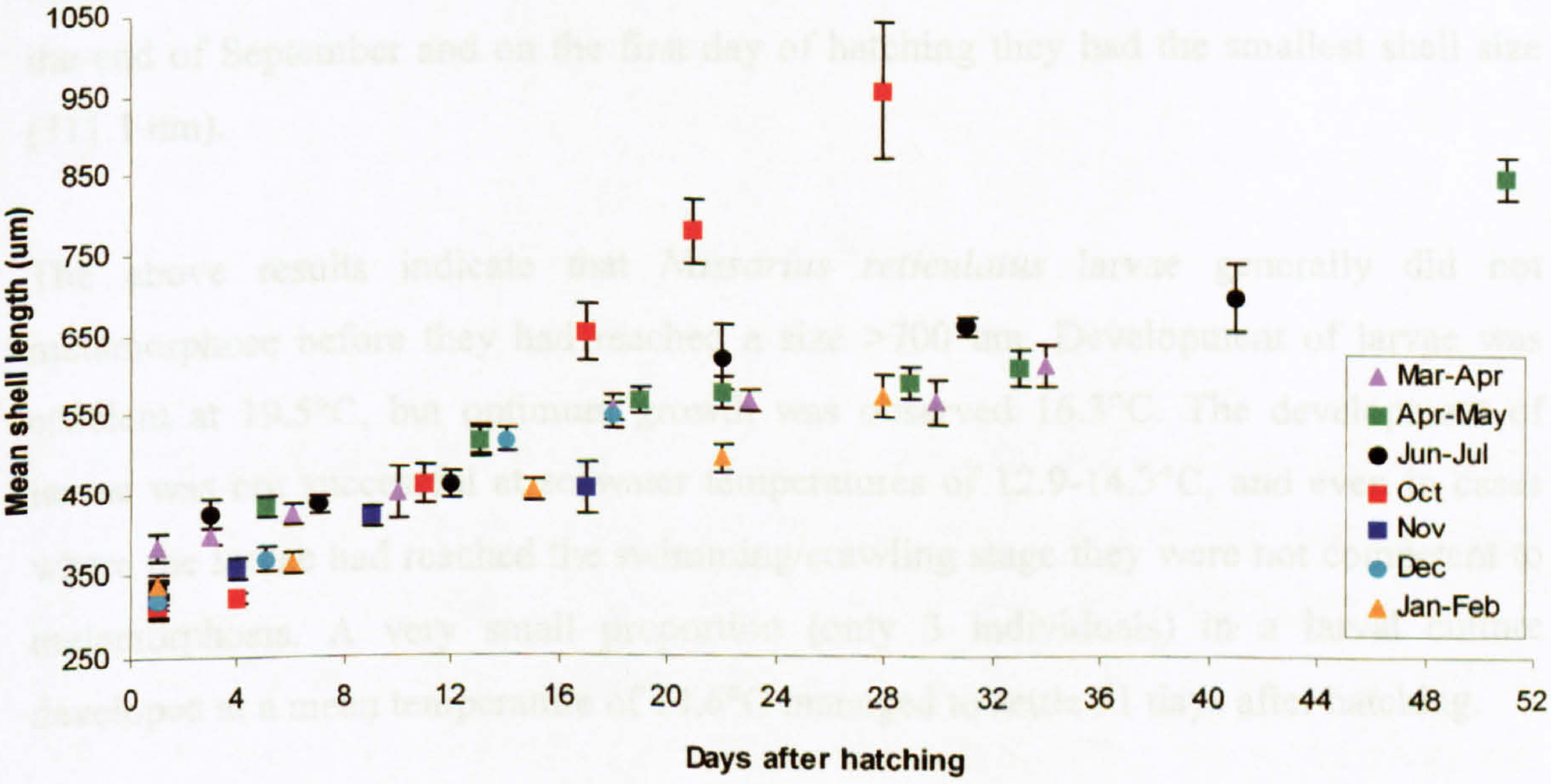
Figure 6.5 Seasonal variation in shell length of larvae recently hatched at ambient temperature, between different months of the year. The points represent the mean shell length (um) of larvae on the first day of hatching and the error bars are the 95% confidence intervals of these means (1.96·SE). The solid line represents ambient temperature (C°) of the seawater under laboratory conditions during the respective months.



6.3.5 Development of larvae under ambient temperature

The increase in shell length of larvae that were hatched at ambient seawater temperature in the laboratory during different months of the year was recorded until the stage of metamorphosis, in order to investigate seasonal differences regarding larval development (figure 6.6). During larval development under ambient temperature the beakers containing the larval cultures were placed on a water-bench, where water of ambient temperature was flowing around them. The temperatures of the larval cultures inside the beaker were about 2°C higher in summer and about 4°C higher during winter, than the actual ambient temperature (shown in figure 6.5). Thus, for example, the temperature in the larval culture beakers during October (16.3°C) was similar to the ambient seawater temperature during September (16.3°C in figure 6.5).

Figure 6.6 Increase of mean shell length (um) of *Nassarius reticulatus* larvae from hatching to metamorphosis during different months of the year. The data points represent the mean shell length (um) of larvae and the error bars are the 95% confidence intervals of these means (1.96·SE).



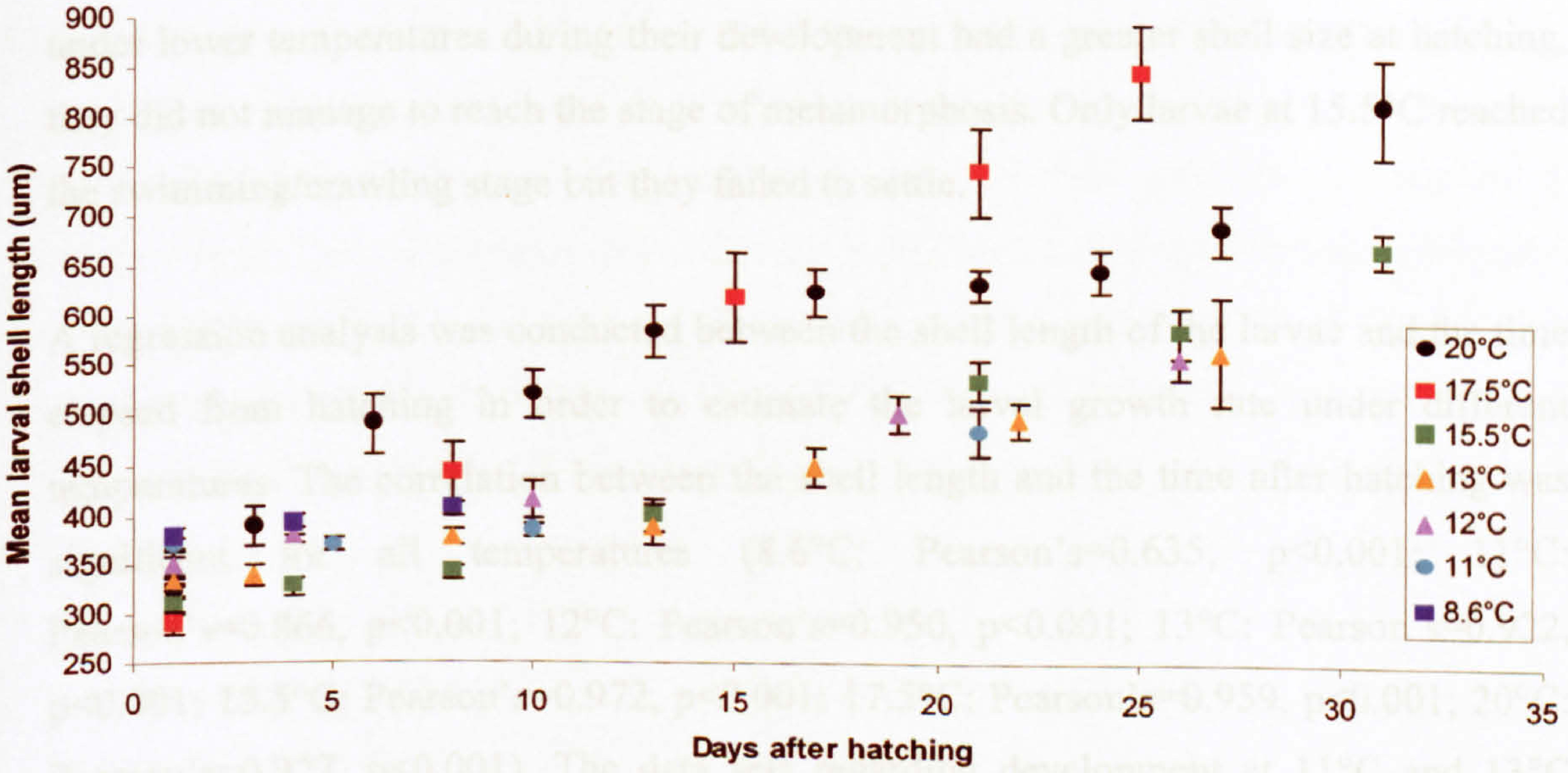
Development of the larvae hatched during November, December and January to February was not completed successfully and the larvae died long before metamorphosis. Larvae developed during November died after 17 days having reached a shell length of 455.6um, during December they died after 18 days with a shell length of 546.7 um, and during January-February they died after 28 days with a shell length of 573.3 um. The mean temperature inside the larval cultures was 14.3°C in November, 13.3°C in December and 13.3°C in January-February. Larvae that developed during March-April were close to metamorphosis (swimming/crawling stage) with a shell length of 613.3 um 34 days after they hatched, although they developed under a relatively low temperature of 12.9°C. However, finally no larvae managed to settle and they all died 5 days later (39 days after hatching). Even though larvae hatched in March had the biggest shell size on the first day of hatching (384.4 um), which might have given them an advantage for better development at low temperatures, they did not achieve settlement. Those larvae developing between April and May (14.6°C) were close to metamorphosis (swimming/crawling stage) with a shell length of 608.9 um after 33 days. However, the culture gradually perished and only 3 larvae were settled after 51 days with a size of 850 um. The larvae developed during June-July, under a relatively high temperature of 19.5°C, reached a shell size of 698 um and settled after 41 days. Optimum growth rate was observed for larvae developed during October under a mean temperature of 16.3°C. These larvae were in the swimming/crawling stage after 21 days with a shell size of 777.8 um, and they finally settled after 28 days having reached a size of 956 um. The larvae which developed during October had hatched at the end of September and on the first day of hatching they had the smallest shell size (311.1 um).

The above results indicate that *Nassarius reticulatus* larvae generally did not metamorphose before they had reached a size >700 um. Development of larvae was efficient at 19.5°C, but optimum growth was observed 16.3°C. The development of larvae was not successful at seawater temperatures of 12.9-14.3°C, and even in cases where the larvae had reached the swimming/crawling stage they were not competent to metamorphosis. A very small proportion (only 3 individuals) in a larval culture developed at a mean temperature of 14.6°C managed to settle 51 days after hatching.

6.3.6 Development of larvae under constant temperature

The development of *Nassarius reticulatus* larvae up until the stage of metamorphosis, hatched in the laboratory under ambient temperature, was studied under 7 different constant temperatures (20, 17.5, 15.5, 13, 12, 11 and 8.6°C). The larvae used in each temperature experiment were hatched under their respective ambient seawater temperatures in the laboratory. The difference between the experiment described in section 6.3.5 is that here the temperature was controlled and constant during the whole larval developmental period. The increase in shell length of larvae from the first day of hatching until metamorphosis under different temperatures is presented in figure 6.7.

Figure 6.7 Development of *Nassarius reticulatus* larvae from hatching until metamorphosis under different constant seawater temperatures. The points represent the mean shell length (um) of larvae and the error bars are the 95% confidence intervals of these means (1.96·SE).



Development of larvae at 8.6 and 11°C was not successful and the larvae did not reach the stage of metamorphosis, or even the stage of swimming/crawling. Larvae, which developed at 8.6°C, attained a size of 406.7 um in 8 days after hatching but all died 5 days later (13 days from hatching). When the larvae developed at 11°C, only 6 individuals survived until the 21st day after hatching, with a shell size of 481.5 um, and they finally died. Larvae developing at 12 and 13°C reached a size of 555.6 um in 26 days and 563 um in 27 days respectively, but they did not manage to metamorphose and died a few days later (32 days after hatching for 12°C and 34 days for 13°C). Larvae which were maintained at 15.5°C reached the swimming/crawling phase 31 days after hatching with a shell size of 666.7 um, but did not manage to metamorphose and the larval culture gradually perished. Larvae developed at 17.5°C reached the stage of swimming/crawling 21 days after hatching at a size of 744.4 um and they succeeded to metamorphose 4 days later (25 days from hatching) at a size of 845 um. Finally, larvae developed at 20°C started crawling after 27 days at a shell length of 686.7 um and similarly managed to metamorphose after 4 days (31 days from hatching) at a size of 808.9 um.

Larval development was optimal at 17.5°C, which was a comparable temperature to the one indicated from the experiments under ambient temperature (16.6°C for October larvae). Growth at 17.5°C was rapid (figure 6.7) and the larvae settled earlier and at a larger size (25 days, 845 um) than larvae developed at a higher temperature of 20°C (31 days, 808.9 um). Although the larvae produced during the colder months and incubated under lower temperatures during their development had a greater shell size at hatching, they did not manage to reach the stage of metamorphosis. Only larvae at 15.5°C reached the swimming/crawling stage but they failed to settle.

A regression analysis was conducted between the shell length of the larvae and the time elapsed from hatching in order to estimate the larval growth rate under different temperatures. The correlation between the shell length and the time after hatching was significant for all temperatures (8.6°C: Pearson's=0.635, $p<0.001$; 11°C: Pearson's=0.866, $p<0.001$; 12°C: Pearson's=0.950, $p<0.001$; 13°C: Pearson's=0.922, $p<0.001$; 15.5°C: Pearson's=0.972, $p<0.001$; 17.5°C: Pearson's=0.959, $p<0.001$; 20°C: Pearson's=0.927, $p<0.001$). The data sets regarding development at 11°C and 13°C were not normal (even after a log transformation), thus regression analysis was conducted only for the remaining 5 temperatures (Normality test 8.6°C: $A^2= 0.627$, $p=0.093$; 12°C: $A^2= 0.618$, $p=0.102$; 15.5°C: $A^2= 0.478$, $p=0.229$; 17.5°C: $A^2= 0.637$, $p=0.090$; 20°C: $A^2= 0.685$, $p=0.071$). A significant regression was found between shell length and time after hatching for the 5 experimental temperatures (8.6°C: $F= 18.9$, $p<0.001$; 12°C: $F= 445$, $p<0.001$; 15.5°C: $F= 1176.3$, $p<0.001$; 17.5°C: $F= 443.1$, $p<0.001$; 20°C: $F= 600.2$, $p<0.001$). The slope estimated from the regression analysis indicated the rate of growth of the larvae (table 6.2).

Table 6.2 Growth rate (±SE) (um·d^{-1}) of larvae during their planktonic life and initial size at hatching (±SE) (um) for development under 5 different constant seawater temperatures.

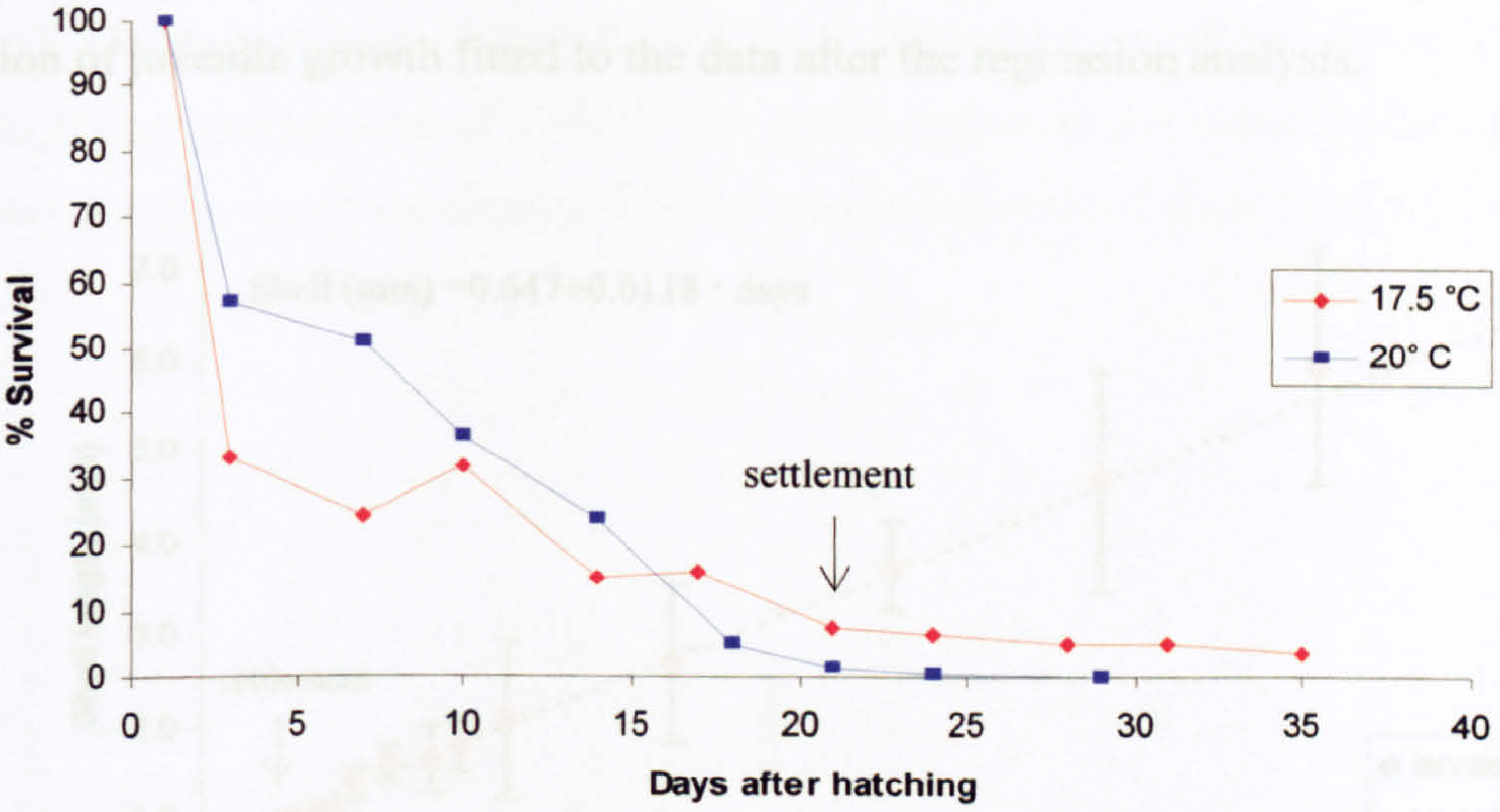
Temperature (°C)	Growth rate (±SE) (um·d^{-1})	Size at hatching (±SE) (um)
8.6	4.1 (±0.9)	377.8 (±5.7)
12.0	8.2 (±0.4)	351.1 (±6.5)
15.5	12.2 (±0.4)	311.1 (±4.7)
17.5	23.0 (±1.2)	291.1 (±6.2)
20.0	13.3 (±0.5)	324.4 (±3.6)

A General Linear Model analysis was conducted in order to compare the regression lines of larval shell length in relation to time after hatching between the different temperatures. The data had equal variances (Bartlett's: T =0.991, p=0.997). A significant difference was found in the initial size of larvae at hatching (intercept: F =30.36, $p<0.001$) and the larval growth rate (slope: F =62.48, $p<0.001$) between the 5 temperatures. The highest growth rate (23.0 um·d^{-1}) was observed for larvae developing at 17.5°C although they had the smallest shell length on the first day of hatching (291.1 um), whilst larvae hatched at larger sizes (377.8 um and 351.1 um respectively) and developed under 8.6°C or 12°C had the lowest growth rates (4.1 um·d^{-1} and 8.2 um·d^{-1} respectively).

6.3.7 Larval survival

Having established (see above) that development of the larvae resulted in successful metamorphosis at 17.5 and 20°C (with the former being the optimum) the percentage survival from hatching to settlement of larvae that were developed under these temperatures was also investigated (figure 6.8). A higher percentage of larvae survived during the 15 first days in larval cultures developing at 20°C, but during the final stages before and after metamorphosis more larvae survived at 17.5°C. In general a very high mortality was observed during the planktonic phases of *Nassarius reticulatus* larvae. Only 8% of the veligers survived until metamorphosis (21 days after hatching) in the culture incubated at 17.5°C, and only 4% of the juveniles survived until the 35th day when the experiment was terminated.

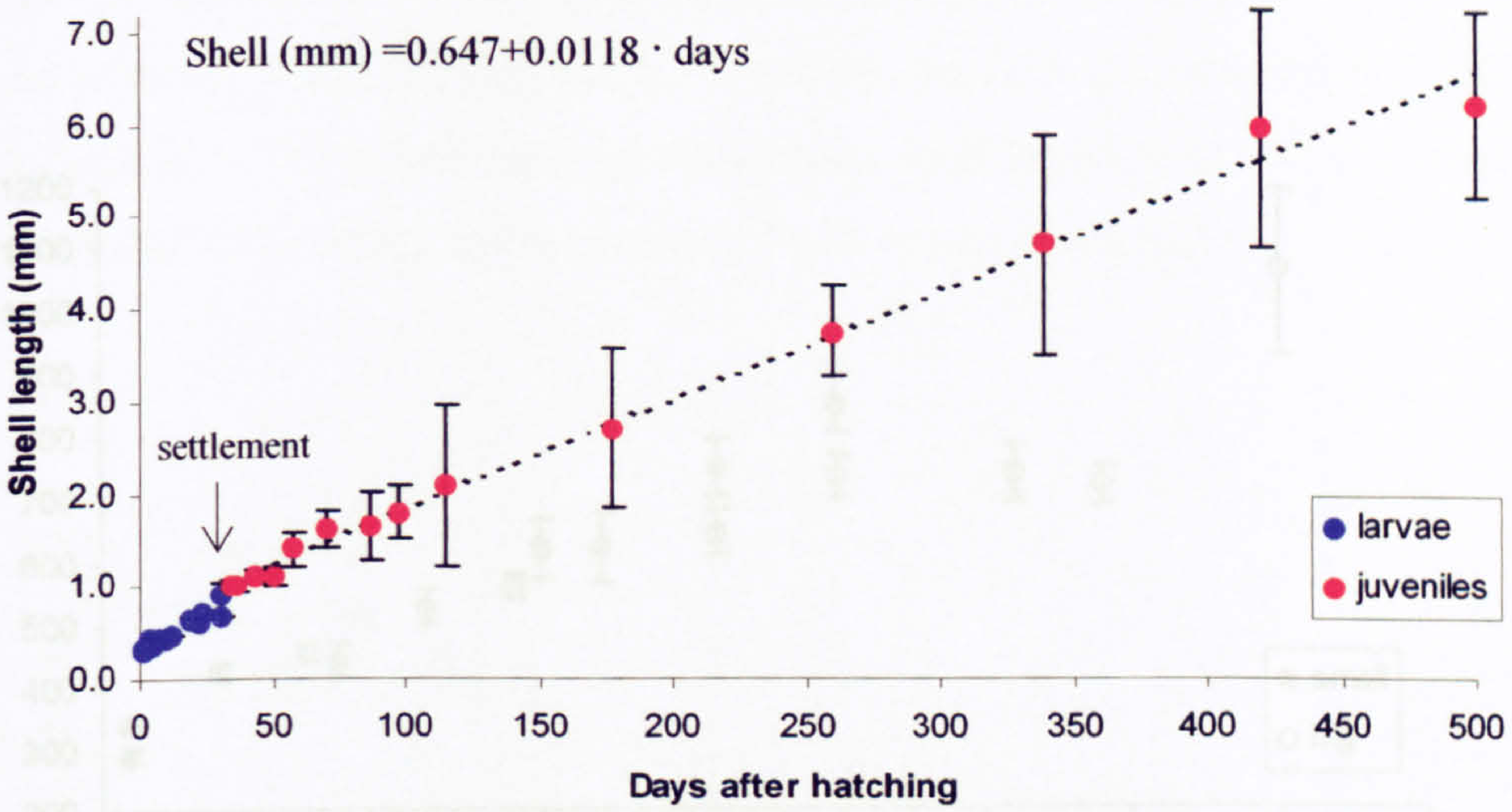
Figure 6.8 Survival (%) of larvae developing at seawater temperatures of 17.5 and 20°C from hatching until settlement and early juvenile stages.



6.3.8 Juvenile development

The development of *Nassarius reticulatus* juveniles was investigated under conditions of ambient temperature from the metamorphosis stage (41 days after hatching) and up to the age of 500 days (figure 6.9). These larvae hatched during June-July 2003 under ambient temperature. The juveniles started scavenging on fish and mussel flesh 178 days after hatching (137 days after settlement). Growth rate was described from the equation of shell length (mm) in relation to age (days after hatching). The data were normal ($A^2 = 0.489$, $p=0.185$), and a significant correlation ($r = 0.996$, $p<0.001$) and regression ($F=1701.7$, $p<0.001$) were found between shell length and time. The growth rate of juveniles under ambient temperature was estimated at 11.8 um·d^{-1}. The growth rate of the respective larvae (June-July) in ambient temperature was 15.2 um·d^{-1} (normal data: $A^2 = 0.297$, $p=0.533$; significant regression: $F=77.92$, $p<0.001$). Thus the growth rate of the benthic juvenile whelks was about 22% lower than the growth rate of the larvae during the planktonic phase.

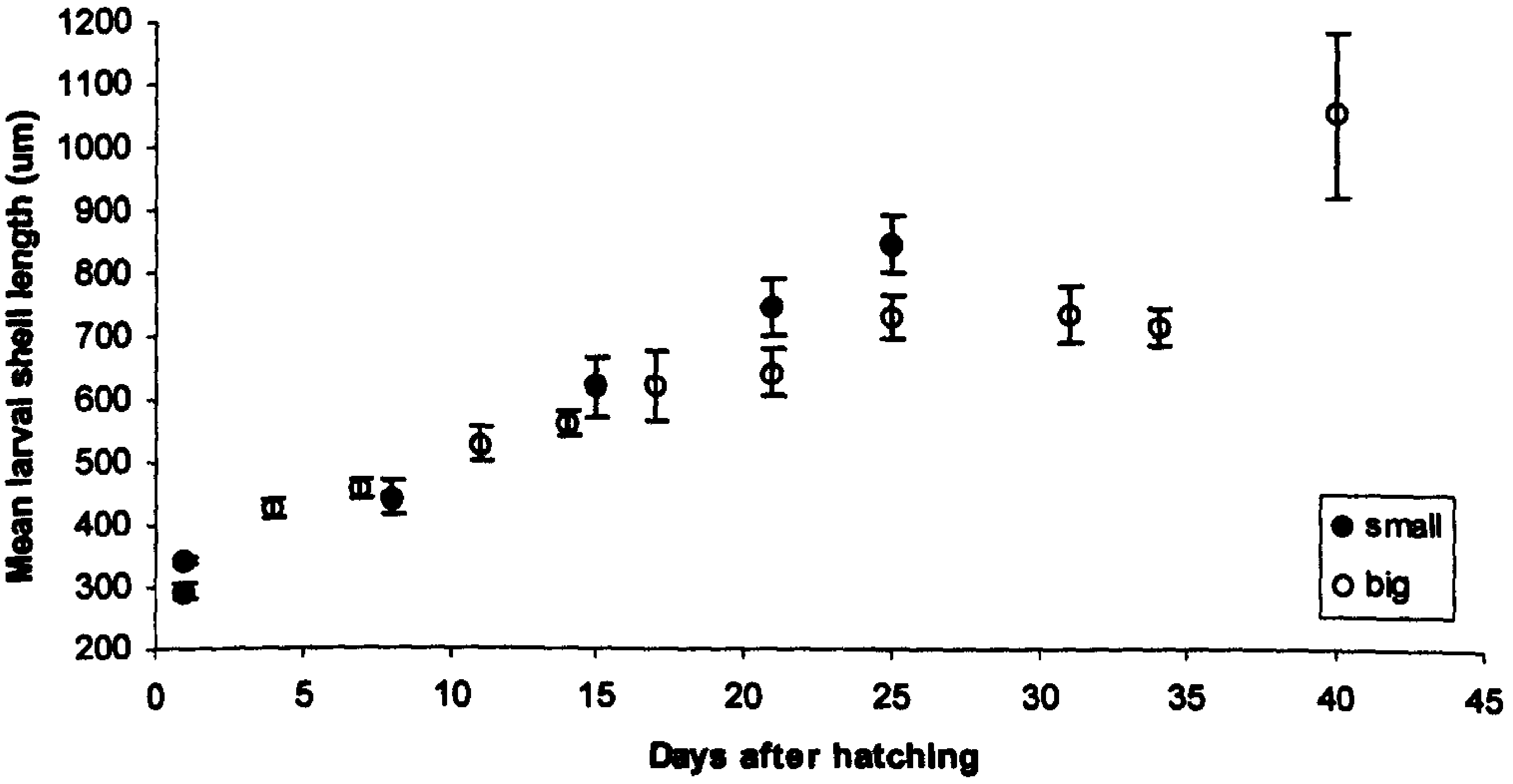
Figure 6.9 Development of *Nassarius reticulatus* juveniles after settlement. The shell length of individuals before settlement (larvae) is also included in the figure. The points represent the mean shell length (mm) of larvae and juveniles, and the error bars are the 95% confidence intervals of these means (1.96·SE). The dashed line represents the equation of juvenile growth fitted to the data after the regression analysis.



6.3.9 Development of "small" and "big" larvae at the same temperature

It has been shown previously (section 6.3.6) that smaller larvae were produced from smaller eggs during the warmer months and that they developed successfully and metamorphosed under an optimum temperature of 17.5°C. Larger larvae were produced, usually during the colder months, but they did not manage to reach the settlement stage. In order to investigate the potential of these larger larvae to settle at higher temperatures a comparison between development of "big" and "small" larvae was performed under the same optimum temperature of 17.5°C (figure 6.10). Small larvae hatched during September (16.3°C) with an initial shell length of 291.1 um, and the bigger larvae hatched in May (12.4°C) with an initial size of 340 um. Both groups of larvae were gradually acclimatised to 17.5°C. The small larvae were at the swimming/crawling stage 21 days after hatching with a shell length of 744.4 um and settled after 4 days (25 days from hatching) with a shell size of 845 um. The bigger larvae were at the swimming/crawling stage 34 days after hatching (715.6 um) and they metamorphosed on the 40th day after hatching with a shell length of 1053 um. Both the small and big larvae managed to metamorphose after incubation at 17.5°C. However, the smaller larvae settled earlier and at a smaller shell length, while the larger larvae had a delayed metamorphosis and settled at a larger size.

Figure 6.10 Development of "small" and "big" *Nassarius reticulatus* larvae under the same optimum temperature of 17.5°C. The points represent the mean shell length (um) of larvae and the error bars are the 95% confidence intervals of these means (1.96·SE).

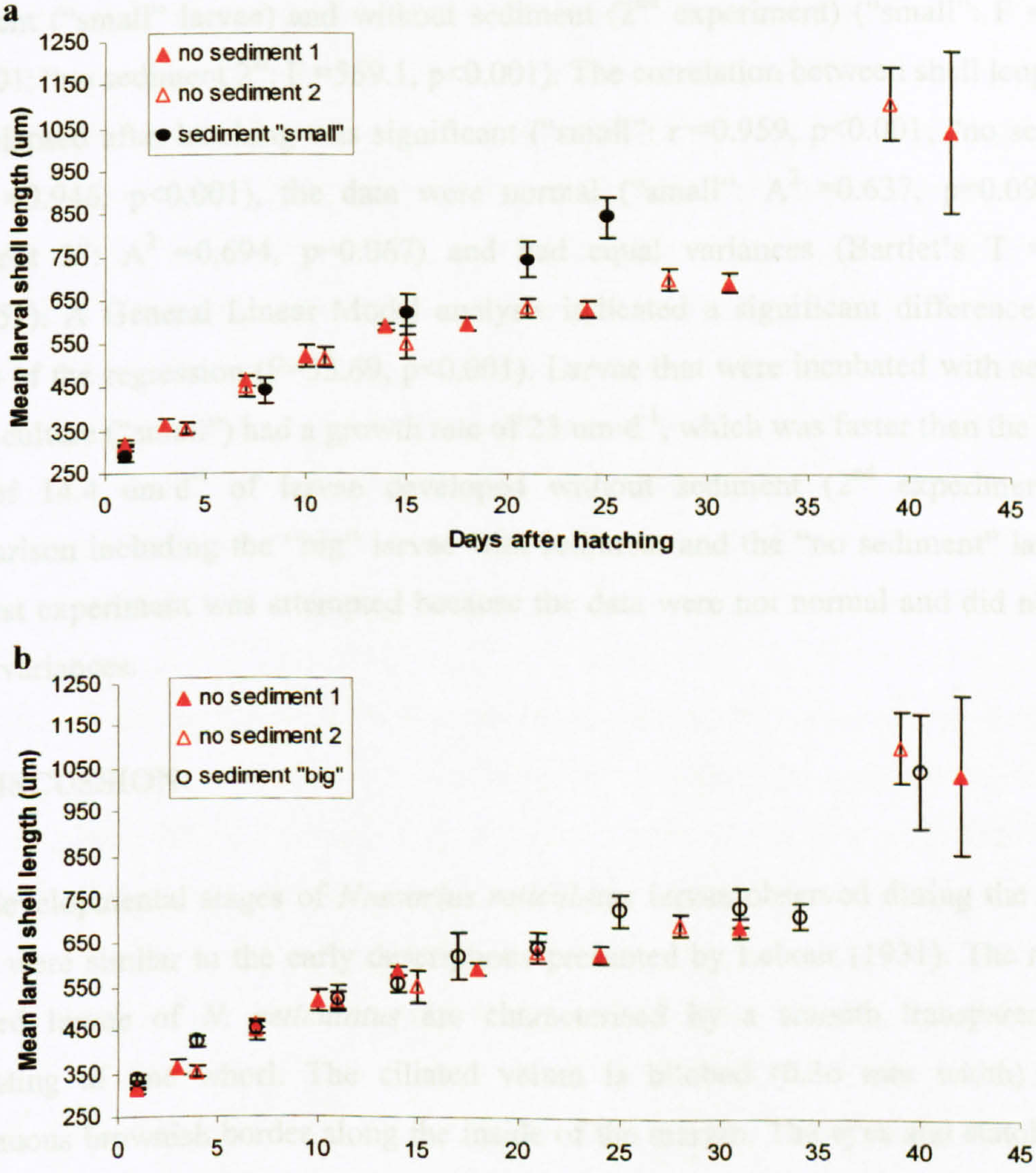


6.3.10 Development with and without addition of sediment

The effect of sediment on development and metamorphosis of larvae was examined by comparing larval development with and without sediment present in the culture beakers. All the larval cultures were maintained at the same temperature of 17.5°C, which was found to be the optimum for larval development. Two experiments without addition of sediment were conducted using larvae that hatched during September with an initial size of 312.5 um and 320 um respectively. The results of the two "no sediment" experiments were compared with the results of the "small" and "big" larvae (section 6.3.9 above) developed at 17.5°C with a supply of sediment (figure 6.11).

The development of the larvae in the two experiments without sediment was similar and in both cases the larvae settled successfully. For the first "no sediment" experiment the larvae were at the stage of swimming/crawling 31 days after hatching with a size of 690 um and they settled about 7 days later (about 38 days after hatching). The size of the metamorphosed larvae was 1045 um, 42 days after hatching. For the second "no sediment" experiment the larvae were at the stage of swimming/crawling 28 days after hatching with a size of 697.2 um and they settled about 7 days later (about 35 days after hatching). The size of the metamorphosed larvae was 1107.5 um 39 days after hatching.

Figure 6.11 Comparison of larval development at constant temperature 17.5°C with and without adding sediment to the larval culture. a) Larvae developed with sediment present had a "small" hatching size and b) larvae developed with sediment present had a "big" hatching size. The points represent the mean shell length (um) of larvae and the error bars are the 95% confidence intervals of these means (1.96·SE).



The "small" larvae that developed with sediment present metamorphosed earlier, 25 days after hatching and at a small size of 845 um. Although the larvae of the two "no sediment" experiments and the "small" larvae all hatched at a similar size during September, the absence of sediment in the larval culture appeared to retard settlement of the veligers (figure 6.11a). However, it was observed that the development of larvae without sediment was similar to the development of "big" larvae with sediment (figure 6.11b). As reported earlier (section 6.3.9) the "big" larvae hatched during May and had a larger initial size, but settled latter than the "small" larvae (settled 40 days after hatching with a size of 1053 um). A similar delay in the metamorphosis of larvae was observed in the case where no sediment was added to the larval culture.

A significant regression was observed regarding the development of larvae with sediment ("small" larvae) and without sediment (2nd experiment) ("small": F =443.1, $p<0.001$; "no sediment 2": F =569.1, $p<0.001$). The correlation between shell length and time elapsed after hatching was significant ("small": r =0.959, $p<0.001$; "no sediment 2": r =0.946, $p<0.001$), the data were normal ("small": A^2 =0.637, p=0.090; "no sediment 2": A^2 =0.694, p=0.067) and had equal variances (Bartlet's T =1.482, p=0.152). A General Linear Model analysis indicated a significant difference in the slopes of the regression (F=53.69, $p<0.001$). Larvae that were incubated with sediment in the culture ("small") had a growth rate of 23 $\text{um}\cdot\text{d}^{-1}$, which was faster than the growth rate of 14.4 $\text{um}\cdot\text{d}^{-1}$ of larvae developed without sediment (2nd experiment). No comparison including the "big" larvae with sediment and the "no sediment" larvae of the first experiment was attempted because the data were not normal and did not have equal variances.

6.4 DISCUSSION

The developmental stages of *Nassarius reticulatus* larvae observed during the present study were similar to the early descriptions presented by Lebour (1931). The recently hatched larvae of *N. reticulatus* are characterised by a smooth transparent shell consisting of one whorl. The ciliated velum is bilobed (0.36 mm width) with a continuous brownish border along the inside of the margin. The eyes and statoliths are present from the very earliest developmental stages. A characteristic tooth was formed as a projection on the outer lip of the shell in order to support the velum. The veligers were able to collect food particles with their ciliated velum while swimming and direct them into the mouth. The algal cells were continuously moved inside the stomach. The cells of food material are subject to mechanical and enzymatic treatment (Fretter & Montgomery, 1968). Vigorous rotation of algal cells against the gastric shield fragments or weakens the cell wall so that digestive secretions can penetrate it (Fretter & Graham, 1994).

The size of the velum increases as the larvae grow, in order to meet the higher needs of food collecting and buoyancy (Fretter & Graham, 1994). The first indications of the 4-lobed velum appeared as an indentation at each side of the velum when the veligers were about 19 days old, and the formation of 4 lobes was completed when the larvae were 31 days old. By that time the foot was perceptibly more developed with stronger colour pigmentation and the shell consisted of more than one whorl. Just before metamorphosis the anterior lobe of the foot expanded into two horns and the veligers were able to swim or crawl at will (swimming/crawling stage). The swimming/crawling stage lasted for 3-4 days and finally the larvae metamorphosed. A swimming/crawling stage that lasted up to 4 days has been described in *Nassarius obsoletus* and *N. vibex* (Scheltema, 1962b). Metamorphosis of *N. reticulatus* was complete when the velum was lost and at the same time the foot and the radula were completely developed, thus juveniles were fully adapted to a benthic lifestyle. Metamorphosis occurred rapidly, which was advantageous given the increased vulnerability of veligers to benthic predation during the transition period from larva to benthic juvenile. At metamorphosis the shell consisted of 2-3 whorls, the shell tooth started to diminish and the tentacles and siphon were well formed.

Nassarius reticulatus larvae were aggregated near the water surface during their initial developmental stages, especially before they were provided with algal culture. After the larvae had been fed for a period of about 2 hours they were more evenly distributed in the water column. As Fretter & Montgomery (1968) suggested, when the larvae had fed and their stomachs were filled, they stopped feeding, sank and remained at a lower level in order to digest their food. Fretter & Montgomery (1968) indicated that the larvae of *N. reticulatus* do not feed for the first 24-72 hours after hatching and they are nourished by their yolk energy reserves, although the larval velum is structurally capable of collecting food particles (Fretter, 1969). During the present study, however, the strong colouration of the larval stomachs just one hour after hatching suggested that the veligers of *N. reticulatus* started feeding on *Rhinomonas reticulatus* and *Pavlova lutheri* immediately after they were released from the egg capsules. The larval stomach becomes pigmented by the presence of algal cells (Fretter & Graham, 1994). Pedersen & Page (2000) similarly found that the cells of *Isochrysis galbana* were found after 1-hour feeding in the stomach of newly hatched larvae of *Polinices lewisii*. Veligers of *Polinices pulchellus* began feeding on *R. reticulatus* within one hour from hatching (Kingsley-Smith *et al.*, 2005).

The swimming pattern of *Nassarius reticulatus* larvae was characterised by a passive sinking phase downwards in the water column, followed by an active swimming phase towards the water surface. The larvae were able to sink slowly through the water when they rapidly withdraw their velum inside the shell (Fretter & Graham, 1994). When the larvae approached the water surface they could briefly swim in a horizontal direction. The larvae swam with their velum orientated up and their shell down, but as they grew older they could turn their velum in several directions. Fretter & Graham (1994) indicated that the natural position of the larva when swimming is with the shell below and the velum above, but during the later stages of development the 4-lobed velum may alter its position in many ways. Several studies have investigated the swimming pattern of molluscan larvae including *Polinices pulchellus* (Kingsley-Smith *et al.*, 2005), *Ostrea edulis* (Cragg & Gruffydd, 1975), *Cerastoderma edule* (Jonsson *et al.*, 1991), *Pecten maximus* (Cragg, 1980) and *Arctica islandica* (Mann & Wolf, 1983). In all cases the larvae swam with their shell directed downwards and their velum expanded upwards. The larvae swam along a vertical axis through upward helical swimming followed by downward gravitational sinking. Asymmetry is responsible for the non-random body orientation, thus resulting in directional swimming patterns (Cragg & Gruffydd, 1975; Jonsson *et al.*, 1991). The dorsal position of the dense calcite shell will result in greater excess density compared to the ventral velar region (Jonsson *et al.*, 1991). An asymmetric density distribution will create a gravitational torque that will tend to return the body to its stable orientation (Jonsson *et al.*, 1991).

Nassarius reticulatus larvae swam near the surface of the water during their initial developmental stages. The swimming ability of the veligers improved with age and their movement became smoother, slower and more controlled. Barile *et al.* (1994) noted that the swimming of *Strombus gigas* veligers became slower as the larvae grew older. As *N. reticulatus* larvae were approaching metamorphosis they spent more time closer to the bottom of the experimental beaker exploring the sediment. Similar alterations of vertical distribution during larval ontogenesis have been observed for several Aplysiidae species (Switzer-Dunlap & Hadfield, 1977), *Polinices pulchellus* (Kingsley-Smith *et al.*, 2005) and *Pecten maximus* (Cragg, 1980). During their early stages larvae spent more time feeding closer to the water surface, and as they approached metamorphosis they began spending more time closer to the bottom. Fretter (1984) also indicated that as the population of larvae aged they spent more time at greater depths.

Recently hatched *Nassarius reticulatus* larvae were not attracted or repulsed by light, but they remained close to the water surface independently of light direction. During the first week of larval life they were not strongly phototactic, though there were some indications that unfed larvae were photopositive and fed larvae were photonegative. A strong photopositive swimming behaviour was observed in unfed larvae of 2-4 weeks old. Most larvae gathered close to the source of light. After the older larvae had been feeding for a 2-hour period they descended to the lower water levels, independently from the light source direction. Upon hatching *Polinices pulchellus* veligers spent most of their time close to the water surface, but were not positively phototrophic in response to a focused light source (Kingsley-Smith *et al.*, 2005). Similarly, Switzer-Dunlap & Hadfield (1977) indicated that after hatching, the larvae of 4 Aplysiidae species tended to swim closer to the water surface, not as a positive phototactic response but probably due to negative geotaxis. Early veligers of *Pecten maximus* tended to accumulate at the surface of the water even in the dark (Cragg, 1980). A negative geotactic swimming behaviour during the early stages would ensure that the larvae are concentrated in regions where food is abundant, and also the more rapid surface water currents would provide most favourable conditions for dispersal (Cragg, 1980). Crisp (1974) indicated that newly hatched invertebrate larvae swim towards the water surface in order to avoid predation by benthic filter feeders.

Eyes and statoliths were observed in *Nassarius reticulatus* larvae during the early developmental stages. Although barometric sensitivity was displayed from the first day after hatching, with a geonegative swimming behaviour, a phototactic behaviour was perceptible only one week after hathcing. According to Fretter & Graham (1994) the eyes of the veliger larvae initially appear as groups of pigmented epithelial cells, which complete their development by the end of the second week, when formation of the crystalline lens and rods occurs. When the development of their eyes is complete they act mainly as simple detectors of light direction, since their image-forming powers are extremely poor (Fretter & Graham, 1994). However, Crisp (1972) noted that photosensitive reactions of adult *N. reticulatus* were not induced by the eyes, cephalic tentacles, metapodial tentacles or the internal epithelium of the siphon, but that they were stimulated by receptors located on the external siphonal epithelium and on the mantle edge. The less clear phototactic behaviour observed by *N. reticulatus* larvae during the first week may be the result of the non-complete development of the photosensitive receptors.

In the present study older *Nassarius reticulatus* larvae (2-4 weeks old) showed a strong photopositive swimming behaviour when they were unfed, but after they had been feeding for a 2-hour period they moved and stayed closer to the bottom of the beaker and displayed a photo-indifferent behaviour. Photopositive swimming behaviour results in the aggregation of planktotrophic larvae where the food concentration is higher, and at the same time protects veligers from benthic predators (Crisp, 1974). The positive phototaxis of *Strombus gigas* larvae was reduced as the larvae grew older (Barile *et al.*, 1994). A photonegative response may evolve when the larvae are closer to metamorphosis, so that veligers have the opportunity to explore the substratum by touch and olfaction and increase their opportunities for settlement (Crisp, 1974; Hughes, 1986). In the present study however, the larvae were not photonegative when they were approaching metamorphosis, and so they would be better characterised as geopositive. They aggregated near the bottom of the beaker irrespective of the light direction. Similarly, the older larvae of *Pecten maximus* tended to accumulate near to the bottom of their containers irrespective of illumination conditions, thus increasing their chances of finding a suitable substrate for settlement (Cragg, 1980).

Nassarius reticulatus larvae demonstrated a strong barometric sensitivity. When larvae of all ages, from newly hatched to the metamorphosis stage, experienced increased pressure they moved towards the water surface. Planktotrophic veligers are not adapted to benthic environments where higher pressures are likely to be encountered, but they prefer to remain closer to the water surface where algal concentration is higher. Even the 2 to 4 week old unfed larvae, which displayed a strong photopositive behaviour under standard atmospheric pressure, moved closer to the water surface when the pressure was increased and ignored the direction of the light. However, older larvae, which spent a longer time away from the surface of the water in order to explore the bottom sediment before their settlement, were less sensitive to pressure increments. Half of the 4-week old larvae (50%) remained close to the sediment when a pressure twice as high as the standard atmospheric pressure was applied (P=2 bar), while under the same pressure 80% of the 3-week old larvae and 100% of the 2-week old or younger larvae moved rapidly towards the water surface.

Similar responses to pressure have been observed in other molluscan species, mostly bivalves. Most *Ostrea edulis* larvae rose off the bottom in response to an increased pressure stimulus, and when the pressure returned to normal many of the larvae immediately sank (Cragg & Gruffydd, 1975). Larvae responded in the same way to pressure changes whether illuminated from the sides or below (Cragg & Gruffydd, 1975). This response may prevent larvae from sinking out of the surface water where their food occurs (Cragg & Gruffydd, 1975). Also Cragg (1980) reported that veligers of *Pecten maximus* rose from the bottom of the experimental jar as a response to increased pressure. Similarly, the veligers of *Arctica islandica* (Mann & Wolf, 1983) and *Mytilus edulis* (Bayne, 1963) exhibited an upward movement following an increase in pressure. *Semibalanus balanoides* nauplii and the furcilaria larvae of *Meganyctiphanes norvegica*, which normally show a positive phototaxis, swam away from the light when the pressure was increased (Rice, 1964). Younger larvae are usually much more sensitive to pressure reductions than older larvae, as has been reported for larvae of *O. edulis* (Cragg & Gruffydd, 1975), *M. edulis* (Bayne, 1963) and *A. islandica* (Mann & Wolf, 1983). Older larvae tend to spend more time near to the bottom of their containers in order to detect a favourable environment for settlement, thus they appear to have reduced barometric sensitivity. Vernberg & Vernberg (1975) indicated that larvae of *Nassarius obsoletus* in the swimming/crawling stage were more resistant to decreased oxygen levels compared with younger larvae. This adaptation may be significant during the late larval developmental stages when veligers move downwards in the water column to less oxygenated water (Vernberg & Vernberg, 1975).

The minimum pressure needed to elicit a pressure response by the larvae of the subtidal *Pecten maximus* was 1.2 bar for 6-17 days old larvae (Cragg, 1980), while the minimum pressure needed to elicit a similar response to larvae of the intertidal *Ostrea edulis* was only 0.2 bar for 11-day old larvae and even less (<0.1 bar) for 5-9 day old larvae (Cragg & Gruffydd, 1975). The threshold change in pressure required to stimulate a significant deviation from the equilibrium swimming pattern was less than 0.5 bar for veligers of the shallow sublittoral *Arctica islandica* (Mann & Wolf, 1983). *Nassarius reticulatus* is an intertidal/shallow sublittoral species, and the larvae (1-day to 4-weeks old) responded to a minimum of 0.2 bar increase in pressure, during the present study. Intertidal and shallow sublittoral species are much more sensitive to pressure reductions because they face an increased danger of sinking and getting trapped in the sediment, while larvae found in deeper water are able to range over a considerable vertical distance and are less sensitive to pressure stimuli (Cragg, 1980).

Planktonic larvae are able to regulate their position in the water column to a certain degree by means of their swimming behaviour, thus they must be able to detect and react to changes in certain environmental variables such as hydrostatic pressure (Cragg & Gruffydd, 1975). Geotaxis is considered as a true perception of the direction of gravity by means of statocyst-like mechano-receptors (Jonsson *et al.*, 1991). Larvae with well developed statocysts are barosensitive and swim upwards when the hydrostatic pressure is increased, in order to maintain their position in the water column (Crisp, 1974). In cases where no statocysts were detected in the early larval stages, geotaxis was considered as a passive consequence of the interaction between gravity and the body asymmetry (Jonsson *et al.*, 1991). Cragg (1980) was not able to detect any statoliths in the larvae of *Pecten maximus* before metamorphosis, thus he indicated that older larvae swim closer to the bottom as a result of a reduction in their swimming effort, rather than a true positive geotaxis. However, in the case of *Nassarius reticulatus*, the statoliths were present during the earliest ontogenetic stages, and the significant pressure responses observed indicated that the larvae were able to actively regulate their distribution within the water column.

Changes in larval responses to environmental cues, such as light and gravity with age alter the swimming patterns and distribution of larvae, thus enhancing the possibilities for detection of a suitable substratum for settlement (Miller & Hadfield, 1986). Ontogenetic changes in light and gravity responses, resulting in different larval swimming behaviours, are crucial for successful settlement and may result from changes in the larval photoreceptors or development of the nervous system (Miller & Hadfield, 1986). According to Miller & Hadfield (1986) variability in age at hatching, age at metamorphic competence and swimming patterns will contribute to a greater dispersal of larvae from the parental area (increased range of geographic distribution) and also from each other (decreased competition between juveniles).

During the present study the average size of larvae on the first day of hatching was greater for larvae hatched in March and April (384.4 um and 375.1 um respectively), whilst the larvae hatched in July, September and October had the smallest average shell length (311.8, 307.4 and 310.4 um respectively). Release of veligers from egg capsules occurs about 1-2 months after egg laying (chapter 5). Therefore the larger larvae, which hatched in early spring, were released from egg capsules that were laid during late winter, when the seawater temperature was lowest. Accordingly, the veligers of a smaller size that were hatched during late summer and autumn were released from egg capsules that were produced during the warmer summer months. In chapter 5 it was shown that larger eggs were included in the larger winter egg capsules of *Nassarius reticulatus*, while the smaller summer capsules contained smaller eggs. The seasonal variations in larval shell size on the first day of hatching were in accordance with the seasonal diversity in the size of eggs. The bigger winter eggs released larvae with a greater shell size on the first day of hatching, while the smaller eggs that were laid during the warmer months resulted in the release of smaller larvae. The larval reserves of *N. reticulatus* are stored in 4 large yolk-rich macromeres, whose size influences morphogenesis until the veliger is hatched (Grachtrup, 1991). Other studies have demonstrated a positive relationship between egg size and size of larvae at hatching, although usually the comparison was between different species. Planktotrophic species (e.g. *Crepidula fornicata, Crepidula plana, Cerithium atratum, Ilyanassa obsoleta*) and non-planktotrophic species (e.g. *Crepidula convexa, Littorina obtusata, Urosalpinx cinerea, Busycon canaliculatum*) with smaller egg sizes released larvae with a smaller size at hatching (Lima & Lutz, 1990). Also Switzer-Dunlap & Hadfield (1977) indicated that Aplysiidae species with smaller eggs produced smaller veligers.

An extended spawning period has been reported for *Nassarius reticulatus* in British waters by Lebour (1945) (February to September), Fretter & Graham (1984) (March to August) and under laboratory conditions in the present study (chapter 5) (November to August). Although *N. reticulatus* veligers were very common in the coastal plankton off Plymouth, mostly during spring and summer, they were often present throughout the year (Lebour, 1937; 1947; Fretter & Shale, 1973). As a result, veligers of *N. reticulatus* may experience a wide range of temperatures during their development from hatching to metamorphosis. The effect of temperature on survival and development of *N. reticulatus* larvae was examined in order to determine variations regarding the duration of planktonic life and the successful metamorphosis of veligers under different seawater temperatures.

The development and the ability for successful settlement were investigated for larvae that were released during different months under ambient temperature in the laboratory. The development of larvae that were hatched during November, December and January-February (average temperature inside the experimental containers 13.3-14.3°C) was not completed successfully and the larvae died long before metamorphosis. Although larvae hatched between March and April (12.9°C) managed to reach the swimming/crawling stage after 34 days (613.3 um), they did not achieve successful metamorphosis. Even though larvae hatched in March had the biggest shell size on the first day of hatching (384.4 um), which might have given them an advantage for better development at low temperatures, they did not manage to settle. Larvae developed during April-May (14.6°C) also attained the swimming/crawling stage after 33 days (608.9 um), but only 3 individuals managed to settle after 51 days having reached a size of 850 um. The June-July larvae (19.5°C) settled 41 days after hatching with a shell size of 698 um, while larvae developed during October (16.3°C) settled after only 28 days having reached a greater size of 956 um. Development of larvae until metamorphosis was efficient at an average temperature of 19.5°C, but optimum at an average temperature of 16.3°C.

Larvae of *Nassarius reticulatus* are competent to metamorphosis when they have reached a shell size close to or >700 um, which can be achieved in 28 to 51 days after hatching depending on the season. Scheltema (1967) indicated that the temperature required for successful metamorphosis can be different from that required for efficient growth. For example, larvae of *N. obsoletus* grew at temperatures above 11.5°C, but they metamorphosed only when the temperature was 16-17°C or higher (Scheltema, 1967). Accordingly, in the present study larvae released at low temperatures during March-May were competent for metamorphosis but never achieved settlement because the temperature was not high enough. Larvae hatched in June-July grew as the temperature was increased and they synchronised their metamorphosis with the higher temperatures occurring at the end of summer. The temperature of seawater in the experimental beakers was optimal for larval development during October (16.3°C). Although the October larvae had the smallest shell size on the first day of hatching (311.1 um) they had the fastest development and achieved successful metamorphosis.

Egg capsules produced during summer, when the reproductive activity of the adults was higher and the seawater temperature was increased, contained eggs of a smaller size, which released smaller larvae during autumn. However, these smaller larvae had a faster growth rate under ambient temperature (16-17°C in autumn) and were more competent for metamorphosis than larvae with a greater size at hatching.

Rearing of larvae under cyclic temperatures in the laboratory has been used in order to simulate the effect of ambient temperature fluctuations on veliger growth and development. Growth rates at cyclic temperatures were intermediate between growth rates at the corresponding constant temperatures for *Crepidula fornicata* larvae (Lucas & Costlow, 1979) and the mud-crab *Rhithropanopeus harrisii* larvae (Christiansen & Costlow, 1975). Cyclic temperatures enhanced survival of the mud-crab *R. harrisii* larvae near the upper temperature limit. However, *C. fornicata* larvae reared under cyclic temperatures had discontinuities in their shell due to inhibition of shell secretion during the adverse part of each cycle (Lucas & Costlow, 1979).

Having established in this current study that temperature was a significant factor affecting larval development, further experiments were conducted under constant temperature in order to estimate variations in larval growth rates, without the confounding effects of fluctuating ambient temperature cycles. Development of larvae under constant temperatures of 8.6°C, 11°C, 12°C and 13°C was not successful and the larvae did not reach the stage of metamorphosis, or even the swimming/crawling stage. The average shell size reached was 406.7 um at 8.6°C (8 days after hatching; 4.1 um·d^{-1}), 481.5 um at 11°C (21 days), 555.6 um at 12°C (26 days; 8.2 um·d^{-1}) and 563 um at 13°C (27 days). Only larvae developed at 15.5°C reached a size of 666.7 um and they were able to swim or crawl at will 31 days after hatching (12.2 um·d^{-1}), though they did not manage to metamorphose. Larvae developed at 17.5°C successfully metamorphosed 25 days after hatching at a size of 845 um (23 um·d^{-1}), while settlement of larvae that were developed at 20°C occurred after a longer period of 31 days at a size of 808.9 um (13.3 um·d^{-1}).

The larvae produced during the colder months of the year had a greater shell size at hatching that could have given them an advantage regarding faster growth and higher survival rates. As George (1994) indicated, when the seastars *Leptasterias epichlora* (lecithotrophic larvae) and *Arbacia lixula* (planktotrophic larvae) produced larger eggs, bigger larvae were released, which had higher survival and growth rates. According to Sargent *et al.* (1987) larger fish juveniles which hatch from larger eggs have lower mortality, faster growth and take less time to become adults. However, during the present study, large larvae released from large eggs during spring did not manage to settle successfully, probably because the low water temperature did not promote all the morphological changes that occur during metamorphosis.

Larvae developed at temperatures from 8.6 to 15.5°C did not settle successfully; however, a greater shell size, a higher growth rate and a longer period of survival were achieved as the temperature increased. The shortest developmental period from hatching to metamorphosis occurred at 17.5°C, during which larvae had the highest growth rate and reached metamorphosis quickly. All the experiments regarding larval development under various ambient or constant temperatures indicated that *Nassarius reticulatus* larvae grew faster and metamorphosed successfully under temperatures of 16-17.5°C. Temperatures lower than 16°C did not promote larval settlement. Larvae developed at temperatures higher than 17.5°C successfully metamorphosed but the growth rate from hatching to metamorphosis was lower. Similarly, Lucas & Costlow (1979) showed that the growth rate of *Crepidula fornicata* larvae increased over a temperature range from 15°C to 20°C, but further increases in temperature from 20°C to 35°C resulted in lower growth rates.

The effect of temperature on the development of gastropod larvae has been extensively reported in the literature, and although temperature ranges are species-specific, in all cases the growth rate of larvae was higher as the temperature was increased. Lima & Pechenik (1985) indicated that high temperatures accelerated growth rates of *Crepidula plana* larvae and reduced the number of days from hatching to metamorphosis. When veligers of *Nassarius obsoletus* were developed under an optimum temperature of 25°C metamorphosis occurred after 10 days, while the developmental period was twice as long (21 days) at 17.5°C, which was the lowest temperature that allowed successful metamorphosis (Scheltema, 1967). The period from hatching to metamorphosis of *Strombus gigas* larvae was reduced as the temperature increased from 24°C to 31°C, while survival and growth rates were increased (Davis, 2000). No signs of metamorphic competence were observed for *S. gigas* larvae when the temperature was 8°C lower than the optimum developmental temperature (Davis, 2000). Pedersen & Page (2000) indicated that larvae of *Polinices lewisii* successfully metamorphosed after 4-5 weeks under laboratory conditions at 20-22°C, while all attempts to rear the larvae at 12°C failed. Tallmark (1980) also indicated that the time from hatching to settlement of *Nassarius reticulatus* larvae was correlated with seawater temperature. Tallmark (1980) reported that larval development in the laboratory was faster at 20°C (25 days) than at 16°C (32 days). In the present study the optimum temperature for development of *N. reticulatus* larvae was between 16 and 17.5°C.

The mortality of *Nassarius reticulatus* larvae was very high during the first developmental stages of the planktonic life. Survival of larvae was higher under temperatures of 20°C for the first 15 days after hatching, but more larvae survived until the final stages of metamorphosis at 17.5°C. Only 8% of the veligers survived from hatching to metamorphosis at 17.5°C, and half of them (4%) survived for a further 14 days after settlement. The heaviest mortality that most species experience occurs during their early developmental period (Vance, 1973). According to Spight (1975b) 90-99% of directly hatched *Thais lapillus* and *T. lamellosa* juveniles died within two months from hatching, and 10-35% of the survivors lived for a further 10 months. Death rates of prosobranch snails are higher during the whole first year than during later years, whether they have planktonic or direct larval development (Spight, 1975b). Thorson (1950) indicated that important causes of mortality during the pelagic veliger phase of marine benthic invertebrates are lack of food, adverse temperature conditions, failure to find the right substrate, transport by currents and predation. Despite the extremely high larval mortality, the enormous numbers of larvae produced are sufficient to ensure population sustainability (Jablonski & Lutz, 1980). Ayers (1956) calculated that only 0.0013% of *Mya arenaria* larvae produced annually needed to survive in order to maintain a clam population in Massachusetts. The larvae of *N. reticulatus* hatched under ambient temperature (June-July) in the present study were successfully raised in the laboratory up to the age of 1.4 years (500 days). The juveniles were finally used to extract their statoliths (see chapter 4). The juveniles were able to scavenge on fish or mussel flesh when they were about 6 months old. The growth rate of juveniles under ambient temperature (11.8 $\text{um}\cdot\text{d}^{-1}$) was about 22% lower than the growth rate of larvae during their planktonic period (15.2 $\text{um}\cdot\text{d}^{-1}$).

Larvae that had the opportunity to develop under optimum temperatures of 16-17.5°C were the ones produced during late summer and autumn at a smaller hatching size. Larger larvae produced during winter did not encounter such temperature ranges. The potential of the bigger winter larvae for successful metamorphosis after development at an optimum temperature of 17.5°C was investigated and a comparison between the development of small and big larvae was conducted. Both the small and big larvae managed to metamorphose after incubation at 17.5°C. However, the smaller larvae settled earlier (25 days) and at a smaller shell length (845 um), than the larger larvae which delayed their metamorphosis for up to 40 days and settled at a much larger size (1053 um). Pechenik *et al.* (1996b) demonstrated, however, that the onset of metamorphic competence in *Crepidula fornicata* larvae was not controlled by the attainment of any particular size.

Larvae successfully metamorphosed 35-38 days after hatching at a shell size greater than 1000 um without sediment present in the larval culture. Larvae with the same size at hatching (referred to above as small larvae), to which sediment was supplied during their final stages of development, metamorphosed earlier, 25 days after hatching, having reached a smaller size of 845 um. The larval growth rate from hatching to metamorphosis was 23 $um \cdot d^{-1}$ when the larvae were supplied with sediment, whereas when no sediment was added the larvae developed with a slower growth rate of 14.4 $um.d^{-1}$. Although *Nassarius reticulatus* larvae metamorphosed even in the absence of sediment, the lack of a suitable substrate for settlement reduced the growth rate of the larvae and delayed metamorphosis.

A similar delay in metamorphosis was observed in larger sized larvae that were produced when the seawater temperature was lower, but they developed at 17.5°C (see above). *Nassarius reticulatus* larvae delayed their metamorphosis for about 10 to 15 days in two cases: a) when larger larvae were produced at low temperatures, even though they were incubated later under optimum temperatures and b) when no sediment was provided during the later developmental stages of the larvae. The fact that larger larvae were released at low temperatures (12.4°C) and were then acclimatised to 17.5°C may have delayed their larval development, even though acclimatisation occurred gradually (~0.5°C daily). In the second case the larvae delayed their metamorphosis due to the fact that no suitable substratum was available for settlement. However, in both cases the period of delay (10-15 days) and the final shell size at which delayed metamorphosis occurred (>1000 um) were similar. Pechenik (1980) indicated that larval development has a genetically fixed end point and that the potential length of the delay period is controlled by the rate at which the larvae progress towards it. Once this point is reached larvae either metamorphose spontaneously despite the absence of metamorphic cues, or they die (Pechenik, 1980). Further investigation regarding the precise nature of cues that promote, or the factors that delay, settlement of *N. reticulatus* larvae is now required.

The presence of a favourable substratum rich in organic content stimulated the metamorphosis of *Nassarius obsoletus* and *N. reticulatus* larvae (Scheltema, 1961; Tallmark, 1980). Veligers of *N. obsoletus* can delay their metamorphosis and increase the pelagic phase by 100% (normally about 14 days) if an appropriate substrate is not present (Scheltema, 1962a). Scheltema (1961) indicated that in the presence of sediment in which juvenile *N. obsoletus* are found in nature, 70% of the larvae metamorphosed successfully, while in treatments without natural sediment only 18.9% of the larvae metamorphosed. By contrast, similar percentages of *Nassarius vibex* larvae were successfully metamorphosed with or without the presence of sediment (Scheltema, 1961). Although veligers of *Polinices pulchellus* were competent to metamorphosis 45 days after hatching, they remained at the swimming/crawling stage for about 6 months in the absence of sediment, and they never metamorphosed (Kingsley-Smith *et al.*, 2005). Remarkable delaying capabilities have been reported for veligers of *Aplysia juliana*, which may sustain a constant shell and tissue mass for over 200 days after having reached a competent size for metamorphosis, when the preferred food (*Ulva*) for adults is absent (Kempf, 1981). Planktotrophic veligers are able to delay their metamorphosis for longer in order to settle on a suitable substrate, compared with lecithotrophic larvae that have limited energy reserves (Hughes, 1986). The relatively low absorption efficiency and the higher respiration rate of *Ilyanassa obsoleta* larvae allows them to develop more slowly and delay their metamorphosis for longer (Pechenik, 1980). Delay in metamorphosis is known primarily from laboratory studies, although limited evidence suggests that at least some individuals of some species delay their metamorphosis in the field (Pechenik, 1990). Species with the greatest habitat specialisation and intertidal species whose larvae are more likely to be swept offshore, show a higher ability to delay their metamorphosis (Pechenik, 1990). The ability to delay metamorphosis and extend planktonic life should increase the potential for dispersal, and therefore increase the probability of encountering a suitable habitat (Pechenik, 1980).

The metamorphosis-inducing properties of sediment are reduced after autoclaving, over-heating or irradiation with ultraviolet light (Scheltema, 1961). These treatments affect the biological and chemical properties of the sediment (quality and quantity of microfauna, microalgae, bacterial flora, organic material), thus, such properties are likely to be responsible for inducing veliger metamorphosis (Scheltema, 1961). Scheltema (1961) indicated that the properties of a sediment favourable for metamorphosis of *Nassarius obsoletus* are mainly generated from water-soluble substances that can be transferred into the seawater. The larvae are able to perceive metamorphosis-inducing substances without being in contact with the sediment, due to their increased chemoreceptive sensitivity (Scheltema, 1961).

In the present chapter the development of *Nassarius reticulatus* larvae from hatching until metamorphosis was studied. A useful method for rearing larvae under laboratory conditions from hatching until successful metamorphosis and settlement of veligers was established. The juveniles were maintained in the laboratory up to the age of 1.4 years. The larvae of *N. reticulatus* underwent a series of ontogenetic changes from hatching until metamorphosis, which resulted in significant morphological and behavioural alterations. The recently hatched larvae were photo-indifferent and geo-negative, while older larvae were photo-positive before feeding and geo-positive after feeding. Increased pressure resulted in the aggregation of veligers closer to the water surface, although older larvae were less sensitive. The size of larvae on the first day of hatching ranged from 307 to 384 um. Larvae released during the colder (winter) months had a larger size at hatching. The larvae were competent for metamorphosis after reaching a shell size of 700 um or greater (~1000um), which was achieved in 25 to 51 days after hatching, depending on temperature, size at hatching or sediment supply. Larvae were not competent for metamorphosis when reared at low temperatures (8-15°C). The optimum temperature for the fastest development and successful metamorphosis was between 16 and 17.5°C. Veligers reared at 20°C also metamorphosed successfully but had a slower growth rate. Mortality of larvae was high from hatching until metamorphosis (92%). Delayed metamorphosis (10-15 days) was observed for the larger larvae produced when the temperature was low but developed under optimum temperature, and for larvae that were not provided with a suitable substratum for settlement.

CHAPTER 7

Development of imposex in *Nassarius reticulatus*

7.1 INTRODUCTION

Antifouling paints based on triorganotin compounds e.g. tributyltin (TBT) and to a lesser extent triphenyltin (TPT) have been used to reduce or prevent the settlement of fouling organisms on ship's hulls since the 1960s (Bennett, 1996). Antifouling paints improve a ship's performance by preventing growth of fouling organisms, such as barnacles and algae, that may decrease the vessel's speed and maneuverability and thus increase fuel consumption (Birchenough *et al.*, 2002). TBT-based antifouling paints are also used on several submerged offshore and coastal marine structures in order to prevent them from metal corrosion (Haderlie, 1984). Unfortunately there is an important environmental cost when TBT leaches from the paint into the marine environment (Birchenough *et al.*, 2002). According to Barreiro *et al.* (2001) TBT contamination is not confined to the vicinity of large trading ports. Disposal of spoils from marinas and harbours may lead to contamination of areas otherwise remote from TBT sources (De Mora *et al.*, 1995; Barreiro *et al.*, 2001). The collapse of oyster farming in western France and southern England during the 1980s, as well as the demise of *Nucella lapillus* populations in south-west England were the results of TBT pollution (Bryan *et al.*, 1986; Alzieu, 1991; Dyrynda, 1992). TBT can be rapidly degraded within the water column via photochemical and biological processes (De Mora & Pelletier, 1997). However, a high percentage is also absorbed onto suspended particulate material and deposited on the sediment, where degradation by microrganisms is an extremely slow process (De Mora & Pelletier, 1997).

Legislation (EC directive 89/677/EEC) banning the use of TBT on boats smaller than 25m was introduced into France in 1982 (Barroso *et al.*, 2002), in Ireland and the United Kingdom in 1987 (Oehlmann *et al.*, 1998), and later applied to most European countries (De Mora, 1996; Steward, 1996). Subsequently, contamination by TBT has declined in many coastal areas (Gibbs & Bryan, 1994; De Mora & Pelletier, 1997) but the problem is still present especially in areas where large vessels predominate (Barroso *et al.*, 2002). The International Maritime Organisation approved a global ban on the use of organotin antifouling paints by 1 January 2003, considering 1 January 2008 to be the last date for having these paints on any vessels (Champ, 2000).

TBT is an immunotoxicant that affects phagocytic activity by interacting and binding with fusion proteins (annexins) in the cell membranes (De Mora & Pelletier, 1997).

Annexins are the proteins that normally bind phospholipids and provide a pathway for communication between the cellular membranes and the cytoplasmic environment (Creutz, 1992). Brick *et al.* (1996) reported that the main effects of TBT on cells of the penis epithelium of *Nassarius reticulatus* were: a) damage of cell organelles, such as mitochondria, Golgi dictyosomes and endoplasmatic reticulum, which are important for oxygen uptake, ATP synthesis, protein synthesis and detoxification in the cells, and b) injury of cell membranes and formation of atypical intercellular spaces that resulted in reduction of the cell integrity within the epithelium layer. Binding of TBT on cellular proteins can disturb steroid metabolism, apparently by inhibiting the activity of cytochrome P450 that is responsible for conversion of testosterone to estradiol 17β (Bettin *et al.*, 1996; De Mora & Pelletier, 1997). The increase in testosterone levels in females can result in the development of a penis (imposex) and subsequent sterilisation (Bettin *et al.*, 1996; Alzieu, 2000). The inhibition of the P450 enzyme system also diminishes the toxicological response of organisms to pollutants (De Mora & Pelletier, 1997). TBT and its degradation products have been found in freshwater and marine fish, birds and marine mammals, mostly concentrated in the liver, the blubber and the muscles (De Mora & Pelletier, 1997).

Triphenyltin (TPT) pollution is not yet very well understood and there is not enough knowledge of its biological effects. TPT derivatives are added in low proportions (8%) to some antifouling paints (Stab *et al.*, 1995) and they are also used in agrochemical applications such as fungicides (Bennett, 1996; Mensink *et al.*, 1996). TPT was classified as an endocrine disruptor (Schulte-Oehlmann *et al.*, 2000), and it was found to induce imposex in *Thais clavigera* (Horiguchi *et al.*, 1997) but not in *Nucella lapillus* (Bryan *et al.*, 1988). Schulte-Oehlmann *et al.* (2000) noted that TPT caused imposex in the freshwater gastropod *Marisa cornuarietis* and reduction of the male penis, and this in turn could result in decreased fecundity or even complete inhibition of spawning in higher concentrations. Exposure of *Nassarius reticulatus* in sediments containing TPT did not cause imposex, but caused impairment of spermatogenesis due to atrophy of the male testis, and also impairment of oogenesis due to inhibition of ovary maturation (Schulte-Oehlmann *et al.*, 2000). Hyperplasia of gills, osphradia and other organs in the mantle cavity of *N. lapillus* indicated a potential carcinogenic effect of TPT (Schulte-Oehlmann *et al.*, 2000).

The phenomenon of imposex was first described in the dogwhelk *Nucella lapillus* by Blaber (1970). Since then organotin compounds leached from anti-fouling paints have been reported to cause imposex in more than 118 gastropod prosobranch species belonging to 63 genera worldwide (Fioroni *et al.*, 1991). A few examples of imposex affected populations that have been intensively analysed include: *N. lapillus* (Bryan *et al.*, 1986; 1988; Gibbs & Bryan, 1986; Gibbs *et al.*, 1987; Oehlmann *et al.*, 1991; Stroben *et al.*, 1992a; Morgan *et al.*, 1998; Quintela *et al.*, 2000; Birchenough *et al.*, 2002), *Ocenebra erinacea* (Gibbs *et al.*, 1990; 1997; Oehlmann *et al.*, 1992), *Ilyanassa obsoleta* (Smith, 1971; 1980; Jenner, 1979; Curtis, 1994), *Hydrobia ulvae* (Barroso *et al.*, 2000), *Littorina littorea* (Barroso *et al.*, 2000; Birchenough *et al.*, 2002), *Ocinebrina aciculata* (Brick *et al.*, 1996; Gibbs *et al.*, 1997), *Hexaplex trunculus* (Gibbs *et al.*, 1997; Terlizzi *et al.*, 1998), *Thais clavigera* (Horiguchi *et al.*, 1997), *Bolinus brandaris* (Ramon & Amor, 2001), *Buccinum undatum* (Mensink *et al.*, 1996; Nicholson & Evans, 1997; Svavarsson *et al.*, 2001; Birchenough *et al.*, 2002), *Urosalpinx cinerea* (Gibbs *et al.*, 1991), *Neptunea antiqua* (Power & Keegan, 2001; Birchenough *et al.*, 2002), *Hinia incrassata* (Oehlmann *et al.*, 1998) and *Nassarius reticulatus* (Stroben *et al.*, 1992a; 1992b; Bryan *et al.*, 1993; Brick *et al.*, 1996; Gibbs *et al.*, 1997; Barreiro *et al.*, 2001; Barroso *et al.*, 2002).

Exposure of natural populations of gastropods at a concentration of as low as 1 ng l^{-1} TBT over long periods can cause imposex (Bryan *et al.*, 1993). Imposex was first defined by Smith (1971) as the superimposition of male sex characters (penis and vas deferens) onto female prosobranchs that may cause sterility in some species. The most common indication of imposex is the development of a bump over the right tentacle in the female, in the position where the male penis appears, which can reach almost the size of the male penis (Smith, 1971). Sometimes a duct starts to develop from the bump and passes towards the opening of the mantle cavity, where the vas deferens can normally be found in males (Smith, 1971). Sections of the gonads of female *Nassarius obsoletus* that developed imposex did not show any sign of male or hermaphrodite tissue (Smith, 1971). The development of the female penis and vas deferens has been correlated with TBT pollution in several studies (Stroben *et al.*, 1992a; 1992b; Bryan *et al.*, 1993; Huet *et al.*, 1995; Barreiro *et al.*, 2001; Barroso *et al.*, 2002). Another indication of imposex is the convolution of the oviduct of *Nassarius reticulatus* that was observed by Stroben *et al.* (1992b) in France, Barreiro *et al.* (2001) in Spain and

Barroso *et al.* (2002) in Portugal. Smith (1980) reported the same phenomenon in *Ilyanassa obsoleta* and considered it as a sign of masculinisation because it resembled the sinuous seminal vesicle of the males. The effect of TBT on the female genital system is not only the formation of a penis and vas deferens, but also a reduction of the albumen, ingestion and capsule gland (Stroben *et al.*, 1992b).

Imposex can cause sterilisation and lead to the local extinction of gastropod species in badly contaminated areas (Birchenough *et al.*, 2002). Sterilisation is caused either by blockage of the pallial oviduct by overgrowth of vas deferens tissue that prevents deposition of egg capsules, or by a split bursa copulatrix and capsule gland that prevents copulation and capsule formation in the oviduct (Gibbs *et al.*, 1990; Stroben *et al.*, 1992b). The first type of sterilisation has been observed in *Nucella lapillus* (Gibbs *et al.*, 1987; Oehlmann *et al.*, 1991), *N. lima* (Short *et al.*, 1989), *N. lamellosa* (Bright & Ellis, 1990) and *Thais haemastoma* (Spence *et al.*, 1990), and the second type has been observed in *Ocenebra erinacea* (Gibbs *et al.*, 1990; Oehlmann *et al.*, 1992) and in *Urosalpinx cinerea* (Gibbs *et al.*, 1991). *Ocinebrina aciculata* is the only species that exhibits both forms of sterilisation (Oehlmann *et al.*, 1996). Initial investigations did not reveal any kind of sterility in *Nassarius reticulatus* (Stroben *et al.*, 1992b; Bryan *et al.*, 1993; Barroso *et al.*, 2000). However, Barreiro *et al.* (2001) reported that a large proportion of female *N. reticulatus* (4-26%) in populations from northwest Spain, which were suffering from advanced imposex (VDS 4+), were sterile and bore masses of aborted egg capsules. Barroso *et al.* (2002) also reported that up to 50% of female *N. reticulatus* in Portugal, which had highly developed stages of imposex (VDS 4), were sterile with several aborted egg capsules within their capsule gland. The aborted egg capsules formed a compact brown mass that gave a darker and enlarged external appearance in the capsule gland (Barreiro *et al.*, 2001; Barroso *et al.*, 2002). Barroso *et al.* (2002) indicated that these sterile *N. reticulatus* showed no sign of vulva blockage by vas deferens proliferation, similar to that observed in *N. lapillus* (Gibbs & Bryan, 1986). However, in some cases the vulva was externally obstructed. Barroso *et al.* (2002) observed that the vulva was covered by a smooth tissue excrescence or by a free solid cylindrical excrescence growing over the vulva. In some cases the vulva was very small and appeared to be constricted by the capsule gland wall (Barroso *et al.*, 2002).

The use of imposex for the estimation of TBT pollution in the marine environment may overcome some of the difficulties related to the chemical measurement of TBT in water:

a) TBT can be effective in concentrations that are very close to the limits of chemical detection, b) large spatial and temporal variations may occur in the concentration of TBT and c) TBT occurs in higher concentrations in the surface layer and in the sediment than in the water column (Evans *et al.*, 1995). Biomonitoring is a low cost technique and a biological meaningful measure for studying the effects of TBT on an ecosystem, at the individual, population and community levels (Barroso *et al.*, 2000).

The main parameters used by several researchers for the evaluation of the degree of imposex are: the mean female penis length index (PLI), the relative penis size index (RPS) or relative penis length index (RPL), the vas deferens sequence (VDS), the average oviduct stage (AOS) and the imposex incidence (%I), which is the percentage of females affected by imposex (Stroben *et al.*, 1992b; Gibbs & Bryan, 1994; Barroso *et al.*, 2000; 2002; Barreiro *et al.*, 2001). The RPS index expresses the mean bulk size (cube of the length) of the female penis as a percentage of the mean bulk size of the male penis (Gibbs & Bryan, 1994). Stroben *et al.* (1992b) and Bryan *et al.* (1993) indicated that the relative penis length (RPL), which is the uncubed RPS, is a better index for *Nassarius reticulatus* since the male penis of the species is very elongated (14-20 mm). The VDS index in *N. reticulatus* is classified according to a scoring system from 0 to 4 proposed by Stroben *et al.* (1992b). Four stages of imposex development (1-4) with two different types in the stages 1 (1a and 1b), 3 (3a and 3b), and 4 (4 and 4+) were distinguished in scanning electron micrographs (Stroben *et al.*, 1992b). Only VDS stages 1b and 3b are characterised by lack of penis formation in *N. reticulatus* (Stroben *et al.*, 1992b). The vas deferens in imposex affected females of *N. reticulatus* develops progressively from the base of the penis towards the vaginal opening (Stroben *et al.*, 1992b). This condition has been observed in *Ilyanassa obsoleta* (Smith, 1981a), *Ocenebra erinacea* (Oehlmann *et al.*, 1992), *Murex brandaris*, *M. trunculus*, *Hinia incrassata* (Oehlmann *et al.*, 1998), *Buccinum undatum* and *Colus gracilis* (Fioroni *et al.*, 1991). Only in *Nucella lapillus* does the development of the vas deferens occur from two centres, one is behind the right tentacle and the second is close to the genital papilla (Gibbs *et al.*, 1987; Oehlmann *et al.*, 1991). The average oviduct stage (AOS) is evaluated by a 3-stage degree of oviduct convolution proposed by Barreiro *et al.* (2001).

The use of the RPL index for assessing the degree of imposex in *Nassarius reticulatus* requires some caution due to the seasonal variation in penis size throughout the reproductive cycle (Barroso & Moreira, 1998). In some species like *Ilyanassa obsoleta* (Jenner, 1979) and *Littorina littorea* (Graham, 1969) the male completely loses its penis at the end of the breeding season, while in *N. reticulatus* the mean length of the male penis is reduced from maturation stage IV to VI (Barroso & Moreira, 1998). Mean male penis length of *N. reticulatus* in north-west Portugal was 11 mm in July (spent stage of gonads) and 17 mm in February (ripe stage of gonads) (Barroso & Moreira, 1998). Stroben *et al.* (1992b) found that the male penis length was maximal from November to March, and at a minimum in late summer (August-September), during the male sexual repose. The length of the female penis, however, did not change during the whole reproductive cycle and was about 4mm (Barroso & Moreira, 1998). Due to the seasonal changes in the male penis length, the RPL index also exhibits seasonal changes (Stroben *et al.*, 1992b). In order to compare the intensity of imposex between different populations using the RPL index, the animals must be at approximately the same phase in their reproductive cycle (Barroso & Moreira, 1998).

The VDS index can estimate more effectively imposex intensity than the RPL index because it remains more or less the same throughout the reproductive cycle (Stroben *et al.*, 1992b; Oehlmann *et al.*, 1998). Barroso *et al.* (2000) suggested that the VDS index is the most important parameter for biomonitoring TBT pollution, because it can estimate the extent of any reduction in the reproductive capability of the females in a population. Stroben *et al.* (1992b) indicated that the VDS index is more representative because it also includes imposex affected females lacking a penis (stages 1b and 3b). According to Stroben *et al.* (1992b) the VDS index showed the best correlation with TBT body burden in comparison to other imposex indices tested. Stroben *et al.* (1992b) suggested that the RPL index can be used as a supplementary index in highly polluted areas, where the VDS index comes into equilibration with the highest value of 4. Later investigations indicated that the AOS index is an even more efficient imposex indicator for severely contaminated areas (Barreiro *et al.*, 2001), because both RPL and VDS reach a plateau for high TBT burdens (Bryan *et al.*, 1993; Barreiro *et al.*, 2001; Barroso *et al.*, 2002). Barroso *et al.* (2002) showed that the RPL and VDS indices reached a plateau for TBT burdens greater that 400 ng Sn g^{-1} dry wt and 700 ng Sn g^{-1} dry wt respectively. Similarly Barreiro *et al.* (2001) indicated that a plateau in RPL and VDS values was observed at TBT body burdens higher than 500 ng Sn g^{-1} dry wt (85-95% for RPL and 4.3 to 4.5 for VDS).

Nucella lapillus was one of the first gastropod species to be used as a bioindicator for imposex pollution (Gibbs *et al.*, 1987), and later it was classified as a very sensitive species, even to low TBT levels (Stroben *et al.*, 1992b; Bryan *et al.*, 1993). In the early 1990s many of the *N. lapillus* populations around southern England, France and Norway had declined or been eliminated due to TBT pollution (Stroben *et al.*, 1992b). *Nassarius reticulatus* showed a relatively lower sensitivity to TBT pollution than *N. lapillus* (Stroben *et al.*, 1992a; Bryan *et al.*, 1993). Penis development in female *N. reticulatus* as a response to TBT pollution was observed at seawater concentrations of 1 ng Sn l^{-1} (=2.5 ng TBT l^{-1}), while in *N. lapillus* female penis development occurred at concentrations below 0.4 ng Sn l^{-1} (=1 ng TBT l^{-1}) (Bryan *et al.*, 1993). The final point of imposex development (VDS) in *N. reticulatus* was 4+, while in *N. lapillus* it was 6 (Stroben *et al.*, 1992b). The imposex impact is stronger for species such as *N. lapillus*, which lack a planktonic larval stage and thus are more susceptible to local extinctions (Bryan *et al.*, 1986). Species such *N. reticulatus*, which have a planktonic veliger stage, exhibit a wider distribution and are also able to recolonise previously polluted areas where the population had been eliminated or severely depleted (Stroben *et al.*, 1992b). Additionally, *N. lapillus* is only present on rocky shores, thus it is not an appropriate species for the estimation of TBT pollution in sandy-bottom areas (Stroben *et al.*, 1992b).

The sediment-dwelling *Nassarius reticulatus* was initially proposed as a bioindicator for TBT pollution by Stroben *et al.* (1992b) and since then many investigators have validated the advantages of using this key-species for monitoring the biological effects of organotin compounds on marine organisms (Bryan *et al.*, 1993; Barroso *et al.*, 2000; 2002). Bryan *et al.* (1993) and Huet *et al.* (1995) considered *N. reticulatus* less sensitive to TBT than *Nucella lapillus* and thus more appropriate for monitoring heavily contaminated areas. Barroso *et al.* (2000) considered *N. reticulatus* to be the most appropriate species for monitoring TBT pollution in the Ria de Aveiro (Portugal) because it presented a full range of imposex levels in response to the existing environmental gradient of TBT, whilst *N. lapillus* exhibited uniform high values of imposex parameters. The VDS index of the more sensitive *N. lapillus* reached a plateau sooner than *N. reticulatus* (Stroben *et al.*, 1992a). Although *N. reticulatus* shows a relatively low sensitivity to TBT compared to *N. lapillus*, the biological concentration factor is 5.5 x 10^4, which is considered very high for the specific pollutant (Stroben *et* *al.*, 1992b). *N. reticulatus* is a moderately sensitive species, compared with the highly sensitive *N. lapillus* or the poorly sensitive *Littorina littorea*, and may therefore provide a better description of organotin pollution around harbours (Barroso *et al.*, 2002). Bryan *et al.* (1993) recommended that *N. reticulatus* could be a good imposex-based TBT indicator in cases of static or increasing TBT concentrations in water, due to its survival in heavily contaminated conditions.

Bryan *et al.* (1987) demonstrated that imposex in *Nucella lapillus* was not reversible. Also Stroben *et al.* (1992a) noted that although the TBT body burden of *Nassarius reticulatus* declined under TBT-free conditions in the laboratory, no evidence of imposex remission (regarding RPL and VDS) was found in this gastropod after 18 months. However, in a later study Bryan *et al.* (1993) indicated that the penis length of imposex affected female *N. reticulatus* was reduced within 6 years after the 1987 restrictions, although the rate of imposex reversibility was very slow. Also Smith (1981a) indicated that imposex in *Ilyanassa obsoleta* declined when individuals were transferred to a less contaminated area. Therefore, in areas where TBT concentrations decreased after the 1982 restrictions, additional TBT analysis of *N. reticulatus* tissues was a far better indicator than the percentage of imposex in the population (Bryan *et al.*, 1993).

Imposex is highly correlated with TBT tissue concentrations (Barroso *et al.*, 2002); however, different relationships have been found for the correlation of imposex degree and TBT body burden in several published studies. For example, *Nassarius reticulatus* females with 200 ng TBT-Sn g^{-1} dry weight exhibited a maximum VDS 4+ and RPL of 70% in Portugal (Barroso *et al.*, 2002), but only about VDS 2 and RPL 3% in Brittany and Normandy (Stroben *et al.*, 1992b), despite the fact that the mean male penis length was about the same in the two regions (12-13 mm). Such discrepancies may be attributed to different analytical methods, (e.g. overestimation of TBT when other triorganotin residues like TPT are present) or to geographical variability of the species response to TBT (Barroso *et al.*, 2002).

The highest concentration of TBT in *Nassarius reticulatus* was found in the kidney, probably due to the direct excretion of TBT via this organ (Stroben *et al.*, 1992b). However, the highest total amount of TBT was found in the digestive gland/gonad complex, due to the greater mass of this organ (Stroben *et al.*, 1992b). The microsomal cytochrome P-450 dependent mixed-function oxidase system (MFO), which is responsible for dealkylation of TBT to DBT and MBT, is localised in the midgut gland (Stroben *et al.*, 1992b). However, TBT body burden indicates only a short-term bioaccumulation, whilst imposex is very slowly reversible in *N. reticulatus* and is a long-term indicator regarding the presence of organotin compounds (Bryan *et al.*, 1993).

Male *Nassarius reticulatus* collected from Brittany and Normandy contained ~89% of the female TBT-Sn body burden (Stroben *et al.*, 1992b). Stroben *et al.* (1992b) found no correlation between male penis length and TBT body burden. They suggested that the higher TBT content in females might be a consequence of greater reproductive effort, resulting in higher food consumption and thus higher TBT accumulation via their contaminated food. Bryan *et al.* (1993), however, indicated that concentrations of TBT were not significantly different between female and male *N. reticulatus*.

Different species may be subject to different TBT levels in the same site, according to their life styles and duration of longevity (Barroso *et al.*, 2000). Sediment-dwelling species, like *Nassarius reticulatus* and *Hydrobia ulvae*, may be directly exposed to higher levels of TBT than *Nucella lapillus*, which is found on rocky habitats (Barroso *et al.*, 2000), because higher concentrations of TBT are absorbed into the sediment (De Mora & Pelletier, 1997). Diet is another main source of TBT contamination, and the TBT body burden is directly related to the trophic level (Oehlmann *et al.*, 1998). It is thought that the dietary accumulation of TBT by *N. reticulatus* is more important than accumulation of TBT directly from the seawater (Stroben *et al.*, 1992a). Stroben *et al.* (1992a) reported that dietary accumulation of TBT in scavenging *N. reticulatus* contributes to more than half of the TBT body burden, although for the predatory *N. lapillus* the percentage is lower. Another important factor is exposure time, which is related to longevity, being 1-5 years for *H. ulvae* (Graham, 1988), 5 to more than 10 years for *N. lapillus* (Gibbs & Bryan, 1994) and 10 to 15 years for *N. reticulatus* (Tallmark, 1980).

The aims of the present study were: 1) To investigate if *Nassarius reticulatus* populations around Anglesey were affected by imposex, 2) to evaluate the sex ratio in different populations, 3) To compare the growth pattern of the female and male penis, 4) to estimate the incidence of imposex (I%), the relative penis length (RPL) and the vas deferens sequence (VDS) in different *N. reticulatus* populations, 5) to examine the populations for the presence of imposex affected females with a convoluted oviduct or with signs of sterility, 6) to evaluate imposex stage of sympatric *Nucella lapillus* populations, 7) to compare the two imposex indicator species, *N. reticulatus* and *N. lapillus*, in terms of relative TBT sensitivity and 8) to evaluate the stage of recovery of the *N. lapillus* populations in Anglesey during the last 14 years.

7.2 MATERIALS AND METHODS

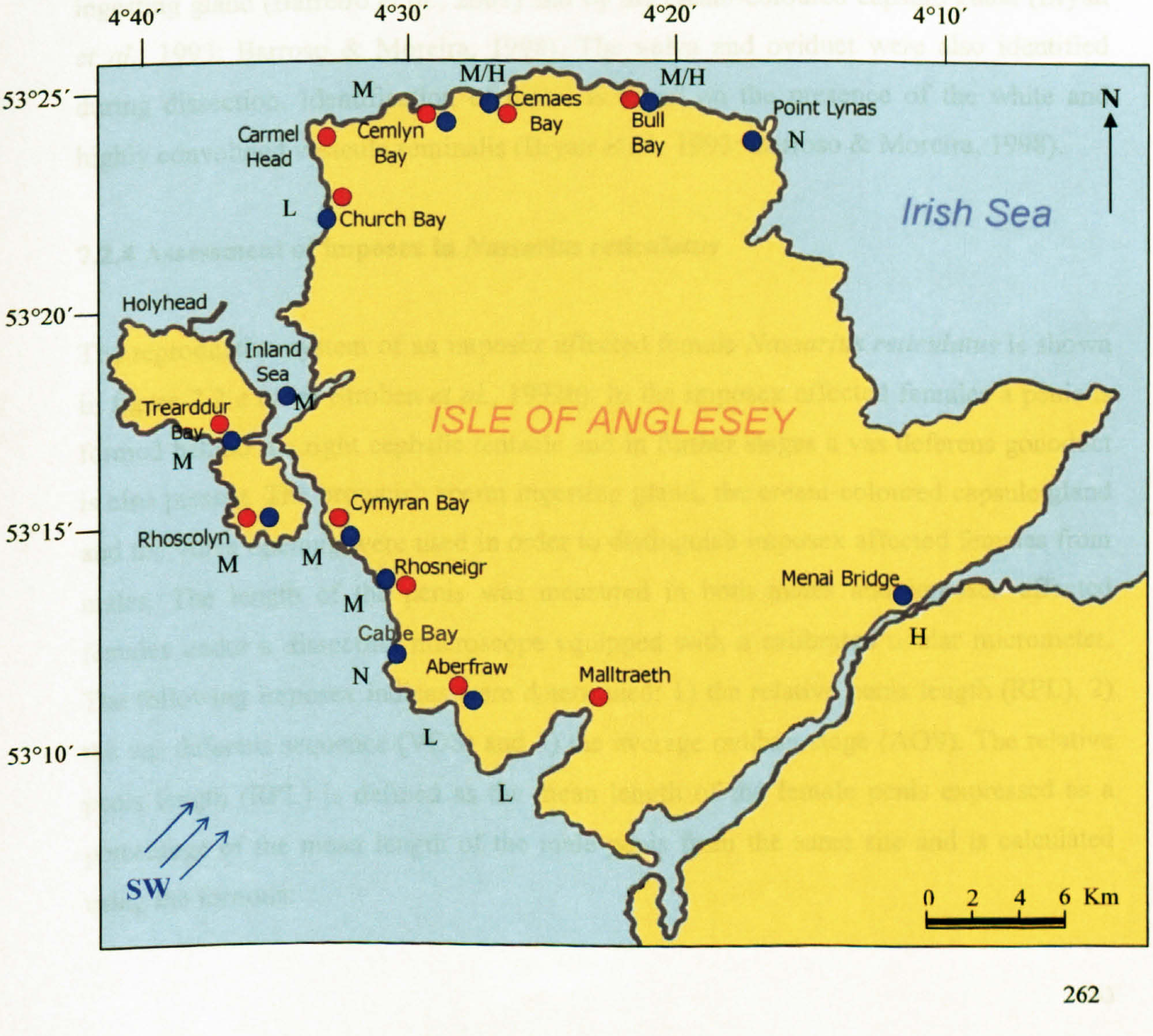
7.2.1 Sampling

The distribution of *Nassarius reticulatus* around the Anglesey coastline was investigated in July 2003 (see chapter 2). During this survey, samples of *N. reticulatus* were collected from 10 subtidal sites (chapter 2.2.1). Also an additional sample of *N. reticulatus* was collected from the intertidal lagoon in Rhosneigr (chapter 3.2.1). A second survey took place in July 2004, during which intertidal samples of adult *Nucella lapillus* were collected, mainly from sites where *N. reticulatus* was present. Some additional sites (Menai Bridge, Cable Bay, Point Lynas), where *N. reticulatus* was absent, were also examined for *N. lapillus* imposex, in order to compare current imposex indices with previous studies. Approximately 50 *N. lapillus* were collected randomly by hand from each site covering a range of the available shell sizes. The levels of imposex were compared between the two different indicator species, *N. reticulatus* and *N. lapillus*. The location of sampling sites is shown in figure 7.1 and a detailed description of the sites can be found in chapter 2.2.2.

7.2.2 Preparation of samples

The shell length (see chapter 2, figure 2.5) of *Nassarius reticulatus* was measured with vernier calipers to the nearest 0.1 mm, after which they were narcotised with 7% $MgCl_2$ in 0.2 μm filtered ultra-violet irradiated seawater for about 30 minutes. Narcotisation with $MgCl_2$ was used in order to obtain comparable results of penis length and imposex stage with previous studies that used the same method. The shell was crushed with a bench vice and the attachment between the columellar muscle and the shell severed. The soft body of the whelk was pinned on a wax layered petri dish with the dorsal side upwards, illuminated using a cold light source and observed under a dissection microscope (Leica Wild M3Z). The mantle was cut and folded back in order to reveal the genitals. Mucus was removed using a cotton bud, and a few drops of 0.1% Toluidine Blue (in 30% ethanol) added in order to improve the contrast of the genitalia against the bulk of the body. The gender and degree of imposex were estimated (see below in 7.2.3 and 7.2.4). After dissection the samples were stored in the freezer for further statolith extraction (see chapter 4).

Figure 7.1 Location of sampling sites around the coastline of Anglesey (North Wales, UK) from where *Nassarius reticulatus* (red symbols) and *Nucella lapillus* (blue symbols) were collected for the examination of imposex. The blue arrows indicate the direction of the south-west prevailing winds. The level of boating activity is also indicated N: none, L: low, M: moderate, H: high.



7.2.3 Identification of gender in *Nassarius reticulatus*

The reproductive system of male and female *Nassarius reticulatus* is presented in figure 7.2 a and b (after Stroben *et al.*, 1992b). According to Ruppert & Barnes (1996) the male reproductive system comprises of a bright red-orange testis and a vas deferens gonoduct, which begins from the testis and transfers sperm to the penis which is located behind the right cephalic tentacle. The vesicula seminalis is the highly convoluted part of the male gonoduct near the testis that is used for sperm storage. The reproductive system of females consists of a yellowish granular ovary and a non-convoluted oviduct, part of which has been modified to an albumen gland and a capsule gland for the production of egg capsules. A brownish sperm ingesting gland is located between the albumen gland and the capsule gland and is used for sperm storage prior to fertilisation or for destroying sperm overflow. As many *N. reticulatus* populations are affected by imposex the penis as a male characteristic is not reliable for gender identification (Barroso & Moreira, 1998). Females can be distinguished by the brownish sperm-ingesting gland (Barreiro *et al.*, 2001) and by the cream-coloured capsule gland (Bryan *et al.*, 1993; Barroso & Moreira, 1998). The vulva and oviduct were also identified during dissection. Identification of males is based on the presence of the white and highly convoluted vesicula seminalis (Bryan *et al.*, 1993; Barroso & Moreira, 1998).

7.2.4 Assessment of imposex in *Nassarius reticulatus*

The reproductive system of an imposex affected female *Nassarius reticulatus* is shown in figure 7.2 c (after Stroben *et al.*, 1992b). In the imposex affected females a penis is formed behind the right cephalic tentacle and in further stages a vas deferens gonoduct is also present. The brownish sperm ingesting gland, the cream-coloured capsule gland and the vulva opening were used in order to distinguish imposex affected females from males. The length of the penis was measured in both males and imposex affected females under a dissecting microscope equipped with a calibrated ocular micrometer. The following imposex indices were determined: 1) the relative penis length (RPL), 2) the vas deferens sequence (VDS) and 3) the average oviduct stage (AOS). The relative penis length (RPL) is defined as the mean length of the female penis expressed as a percentage of the mean length of the male penis from the same site and is calculated using the formula:

$$RPL = \frac{mean\ length\ of\ female\ penis}{mean\ length\ of\ male\ penis} \cdot 100$$

Figure 7.2 Schematic representation of the reproductive system of *Nassarius reticulatus*. a) male, b) female and c) imposex affected female. Abbreviations: ag: albumen gland, at: atrium muscular vestibulum, bc: bursa copulatrix, cg: capsule gland, di: duct of ingestion gland, gpd: gonopericardial duct, ig: ingestion gland, m: transversal musculature, o: ovary, od: oviduct, omc: opening into the mantle cavity, op: opening of the penis, p: penis, pd: penis duct, pvd: pallial vas deferens, rvd: renal vas deferens portion, s: sphincter, t: testis, v: vagina, vc: ventral channel, vd: vas deferens vo: vaginal opening (vulva), vs: vesicula seminalis (from Stroben *et al.*, 1992b).

The VDS index was calculated as the average imposex stage for a population according to the classification scheme presented by Stroben *et al.* (1992b) (figure 7.3).

Stage 1a: tiny penis without a penis duct behind the right tentacle

1b: no penis, but a short vas deferens tract behind the right tentacle.

Stage 2: penis with closed or closing penis duct behind the right tentacle.

Stage 3a: penis with penis duct and an incomplete tract of the vas deferens that is growing towards the vulva (vaginal opening)

3b: penis missing, vas deferens is running continuously from the right tentacle over the bottom of the mantle cavity up to the vulva

Stage 4: penis with a penis duct and a continuous vas deferens from the penis up to the vulva. Often the vas deferens does not join the vulva but passes next to it (Barreiro *et al.*, 2001; Barroso *et al.*, 2002).

Stage 4+: the vas deferens passes the vulva and runs into the ventral channel of the capsule gland. In some cases the vas deferens runs parallel to the gland instead of joining it (Barroso *et al.*, 2002). For estimation of the population average VDS index the 4+ is presented as a numerical value of 4.5 (Barreiro *et al.*, 2001) or 5 (Barroso *et al.*, 2002) in order to discriminate between highly contaminated stations.

Barreiro *et al.* (2001) indicated that another character that can be used as an imposex index is the degree of oviduct convolution. When the normally straight thin oviduct becomes convoluted it resembles the male vesicula seminalis. Convoluted oviducts were found mainly in female *Nassarius reticulatus* that displayed advanced imposex (Barreiro *et al.*, 2001). The oviduct stage was classified according to the scheme presented by Barreiro *et al.* (2001): a normal straight oviduct is characterised as stage 0, a slightly sinuous oviduct is stage 1 and a clearly convoluted oviduct is stage 2. Imposex incidence (I %) was calculated as the percentage of females affected by imposex in each *N. reticulatus* population (Barroso *et al.*, 2002). In addition all the imposex affected females were examined for the presence of aborted egg capsules inside the capsule gland, and for potential vulva blockage.

Figure 7.3 General scheme for imposex evolution in prosobranchs (from Stroben *et al.*, 1992b). Imposex stages of *Nassarius reticulatus* are in boxes. Abbreviations as in figure 7.2. In addition: ac: aborted capsules, gp: genital papilla, obc: open bursa copulatrix, ocg: open capsule gland, ocv: occlusion of the vulva, pr: prostate, te: tentacle, vdp: vas deferens passage into capsule gland, vds: vas deferens section.

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7.2.5 Identification of gender in *Nucella lapillus*

The presence of a penis is not a reliable character for recognition of male *Nucella lapillus*, since the penis length of the imposex affected females may approach that of males (Gibbs *et al.*, 1987). The reproductive system of males and females *N. lapillus* has been described by Gibbs *et al.* (1987) (figure 7.4). Males can be recognised by the presence of the prostate gland, which is mustard-yellowish in colour. Easily recognisable characters in females are the creamy-white coloured capsule gland and the brownish sperm ingesting gland, located immediately posterior to the capsule gland.

Figure 7.4 Reproductive system of *Nucella lapillus*: a) male and b) female. Abbreviations: ag: albumen gland, cg: capsule gland, cm: collumella muscle, dg: digestive gland, f: foot, hg: hypobranchial gland, k: kidney, me: mantle edge, o: operculum, ov: ovary, p: penis, pr: prostate, rg: rectal gland, rt: right tentacle, sg: sperm ingesting gland, t: testis, vd: vas deferens (from Gibbs *et al.*, 1987).

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7.2.6 Assessment of imposex in *Nucella lapillus*

The imposex indices determined for *Nucella lapillus* were the relative penis size (RPS) and the vas deferens sequence (VDS). The imposex incidence (I %) was also calculated as described above for *Nassarius reticulatus*. Relative penis size (RPS) is defined as the mean bulk of female penis expressed as a percentage of the mean bulk of the male penis from the same site, where bulk is the cubed penis length (Gibbs *et al.*, 1987). The RPS index is calculated using the formula:

$$RPS = \frac{(mean\ length\ of\ female\ \ penis)^3}{(mean\ length\ of\ male\ \ penis)^3} \text{ x } 100$$

The VDS index was calculated as the mean imposex stage for a population according to the classification scheme presented by Gibbs *et al.* (1987) (figure 7.5).

Figure 7.5 Imposex stages in *Nucella lapillus* based on vas deferens sequence (VDS). Abbreviations: a: anus, b: blister, cg: capsule gland, gp: genital papilla, n: nodule, p: penis, v: vulva, vd: vas deferens (from Gibbs *et al.*, 1987).

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Stage 0: the unaffected female has no apparent male characteristics (normal)

Stage 1: a short proximal vas deferens tract begins at the opening of the vulva (figure 7.5-1).

Stage 2: Formation of a small penis, often as a small sigmoid ridge, behind the right tentacle (figure 7.5-2).

Stage 3: The small penis becomes measurable with a short distal penis duct growing towards the vas deferens portion arising from the vaginal opening (figure 7.5-3).

Stage 4: The penis is larger and similar to the male penis shape and size. The two sections of the vas deferens, originating from the genital papilla and the base of the penis, become fused into a continuous tube (figure 7.5-4).

Stage 5: The penis is large. The epithelium of the vas deferens proliferates causing a blister-like protuberance (figure 7.5-5A) and forms nodules occluding the vulva opening (figure 7.5-5B).

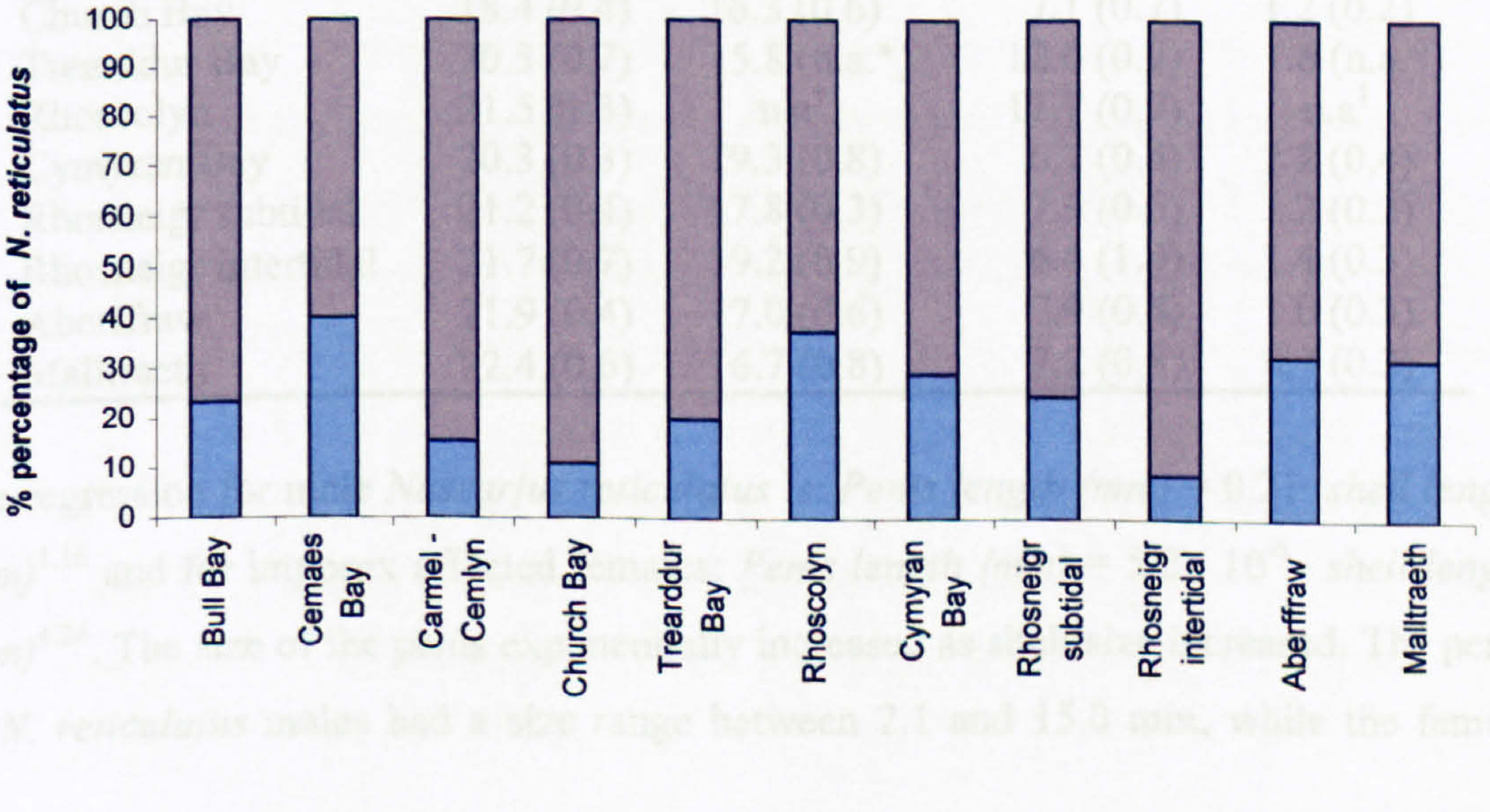
Stage 6: The lumen of the capsule gland is filled with aborted egg capsules that initially are transparent and later form a dark brown mass (figure 7.5-6 A and B)

7.3. RESULTS

7.3.1 Sex ratio of *Nassarius reticulatus*

The number of female *Nassarius reticulatus* was consistently higher than the number of males in samples collected from the 11 different sites around Anglesey (figure 7.6). The percentage of females ranged from 60% at Cemaes Bay to 91.2% in the intertidal lagoon at Rhosneigr. A chi-square comparison was conducted in order to investigate whether there were significant differences in the sex ratios between the sites. The sex ratio was similar between 10 of the 11 sites examined (x^2=1.115, p=0.291). The average sex ratio was 73.5% females and 26.5% males (3:1). Only the intertidal lagoon at Rhosneigr, which had the highest percentage of females, had a significantly different sex ratio from all the other sites (x^2=9.820, p=0.007).

Figure 7.6 The sex ratio of *Nassarius reticulatus* from 11 different sites around Anglesey. The percentage of females is represented by the grey bars and the percentage of males is represented by the blue bars.



7.3.2 Penis measurement in *Nassarius reticulatus*

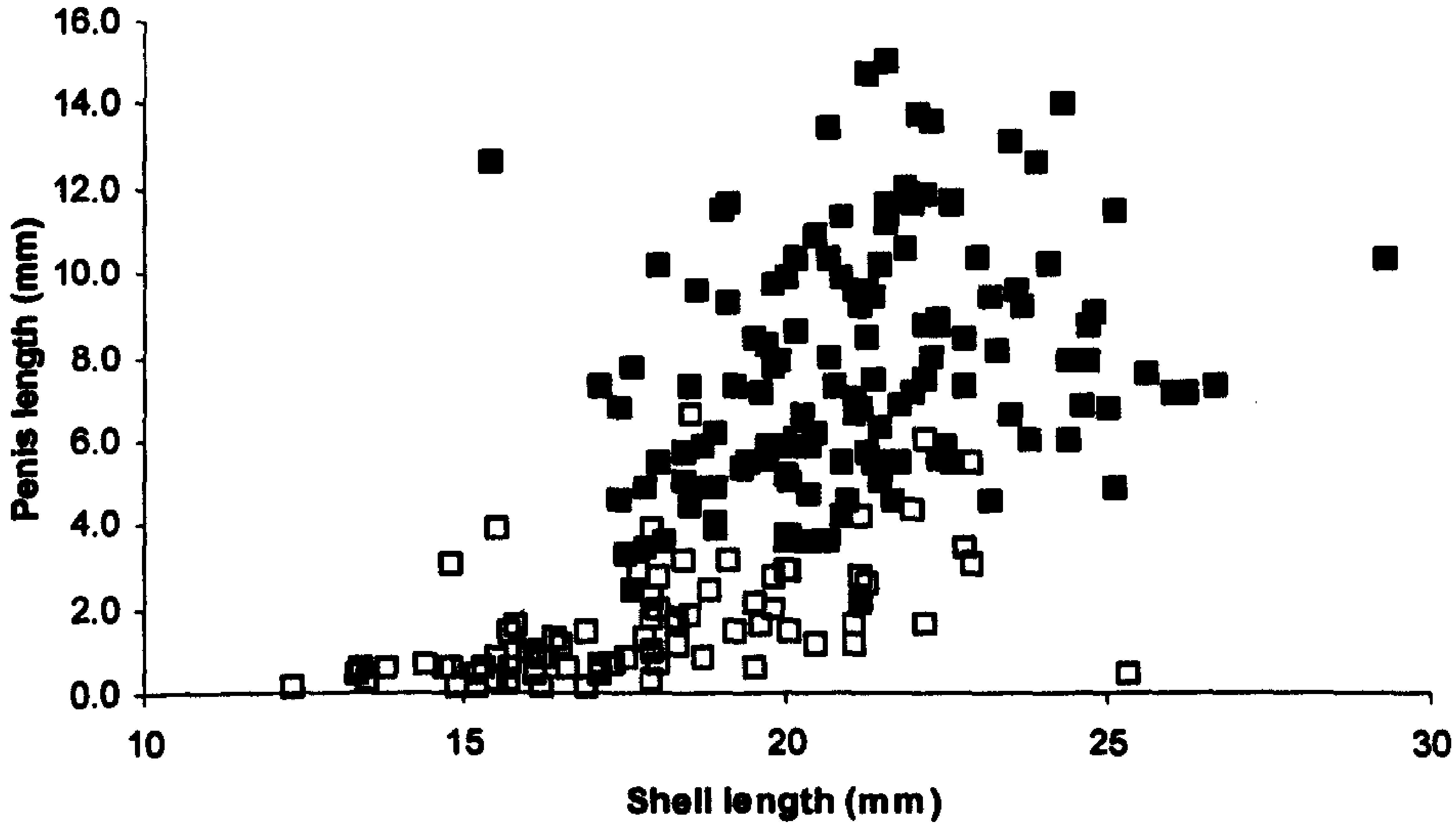
The shell and penis length of males and imposex affected females are presented in table 7.1. In order to investigate the growth of the penis in relation to shell size in males and females, a regression analysis was conducted. Due to the limited number of gastropods collected from some sites the shell and penis length data from all sites were grouped for the regression. A significant correlation was found between shell length and penis length for *Nassarius reticulatus* males ($r=0.280$, $p=0.001$) and imposex affected females ($r= 0.554$, $p<0.001$). A logarithmic transformation was applied on the data in order to meet the criteria of normality (males: $A^2=0.419$, $p=0.324$; females: $A^2=0.682$, $p=0.072$). The regression between shell length and penis size for males and imposex affected females was significant (males: $F=15.14$, $p<0.001$; females: $F=49.33$, $p<0.001$) and it is graphically presented in figure 7.7.

Table 7.1 Mean shell length (mm) (±SE) and mean penis length (mm) (±SE) of *Nassarius reticulatus* males and imposex affected females from 11 different sites around Anglesey. (n.a.*: not-applicable because only one female with imposex was found, and n.a†: not-applicable because all imposex affected females were at VDS stage 1 and the penis was not measurable).

Sites	**Shell length (mm) (±SE)**		**Penis length (mm) (±SE)**	
	male	**female**	**male**	**female**
Bull Bay	20.0 (2.3)	15.7 (n.a.*)	12.7 (0.5)	1.4 (n.a.*)
Cemaes Bay	20.6 (0.9)	14.6 (0.6)	12.9 (1.2)	2.5 (1.0)
Carmel-Cemlyn	20.9 (0.5)	20.0 (0.6)	7.5 (0.6)	3.6 (0.6)
Church Bay	18.4 (0.4)	16.3 (0.6)	7.1 (0.7)	1.2 (0.2)
Trearddur Bay	20.5 (0.7)	15.8 (n.a.*)	12.0 (0.7)	1.6 (n.a.*)
Rhoscolyn	21.5 (1.3)	n.a†	11.7 (0.7)	n.a†
Cymyran Bay	20.3 (0.3)	19.3 (0.8)	6.1 (0.4)	2.2 (0.4)
Rhosneigr subtidal	21.2 (0.4)	17.8 (0.3)	7.9 (0.5)	1.2 (0.2)
Rhosneigr intertidal	21.7 (0.7)	19.2 (0.9)	6.4 (1.4)	1.4 (0.3)
Aberffraw	21.9 (0.4)	17.0 (0.6)	7.9 (0.4)	1.0 (0.3)
Malltraeth	22.4 (0.6)	16.7 (0.8)	7.2 (0.5)	0.7 (0.2)

The regression for male *Nassarius reticulatus* is: *Penis length (mm)* = $0.21 \cdot$ *shell length (mm)*$^{1.16}$ and for imposex affected females: *Penis length (mm)* = $5.2 \cdot 10^{-6} \cdot$ *shell length (mm)*$^{4.26}$. The size of the penis exponentially increased as shell size increased. The penis of *N. reticulatus* males had a size range between 2.1 and 15.0 mm, while the female penis length ranged from 0.1 to 6.6 mm. The unequal variances (Bartlett's T= 0.209, $p<0.001$) between the male and female data indicated that penis growth is not similar between the two genders.

Figure 7.7 Relationship between shell length (mm) and penis length (mm) in males (■) and females (□) *Nassarius reticulatus* from all sampling sites.



7.3.3 Imposex in *Nassarius reticulatus*

After determining the number of males, females and imposex affected females in each of the 11 Anglesey populations, the imposex incidence (I%), the relative penis length (RPL) and the vas deferens sequence (VDS) were estimated (table 7.2). The percentage of *Nassarius reticulatus* females affected by imposex (I%) for the 11 different sites around Anglesey is presented in figure 7.8. A chi-square analysis was conducted in order to compare the percentage of imposex affected females at the different sites (table 7.3). The highest imposex incidence was found in Cemaes Bay (83.3%), which is located on the north coast of Anglesey. Cemaes Bay is an area characterised by moderate to high boating activity. A small port is located in Cemaes Bay where several fishing and leisure boats are anchored. A relatively high imposex percentage was also found in the vicinity of Carmel Head-Cemlyn Bay (42.4%), which has a moderate boating activity. A high percentage of imposex affected females (40%) was also found in the area of Church Bay, which is located on the north-west coast of Anglesey. The area is characterised by low boating activity but the relative proximity to Holyhead harbour may be responsible for the increased imposex incidence observed.

Table 7.2 Imposex indices for *Nassarius reticulatus* from 11 sites around Anglesey. The percentage of females with imposex (I%), the relative penis length (RPL) and the vas deferens sequence (VDS) (mean, range and standard error) are presented for each site. The numbers of males, females and imposex affected females are also indicated. (n.a.*: not-applicable because only one female with imposex was found, n.a.†: not-applicable because all imposex affected females were at VDS stage 1 and no penis was formed).

Sites	**Number of**			**I %**	**RPL%**	**VDS**		
	♂	♀	imposex			**mean**	**range**	**SE**
Bull Bay	3	10	1	10.0	11.3	3.0	n.a.*	n.a.*
Cemaes Bay	4	6	5	83.3	19.5	2.4	1-4	0.6
Carmel-Cemlyn	6	33	14	42.4	48.2	3.3	1-4	0.3
Church Bay	5	40	16	40.0	16.6	3.1	1-4	0.3
Trearddur Bay	3	12	1	8.3	13.3	3.0	n.a.*	n.a.*
Rhoscolyn	3	5	1	20.0	n.a.†	1.0	n.a.*	n.a.*
Cymyran Bay	31	77	10	13.0	35.5	3.3	3-4	0.15
Rhosneigr subtidal	21	65	9	13.8	14.8	3.2	3-4	0.2
Rhosneigr intertidal	5	52	10	19.2	29.7	2.9	2-4	0.2
Aberffraw	36	75	16	21.3	13.0	3.6	3-4	0.1
Malltraeth	20	43	8	18.6	9.2	2.9	2-3	0.1

Figure 7.8 The percentage of *Nassarius reticulatus* females affected by imposex at 11 sites around Anglesey.

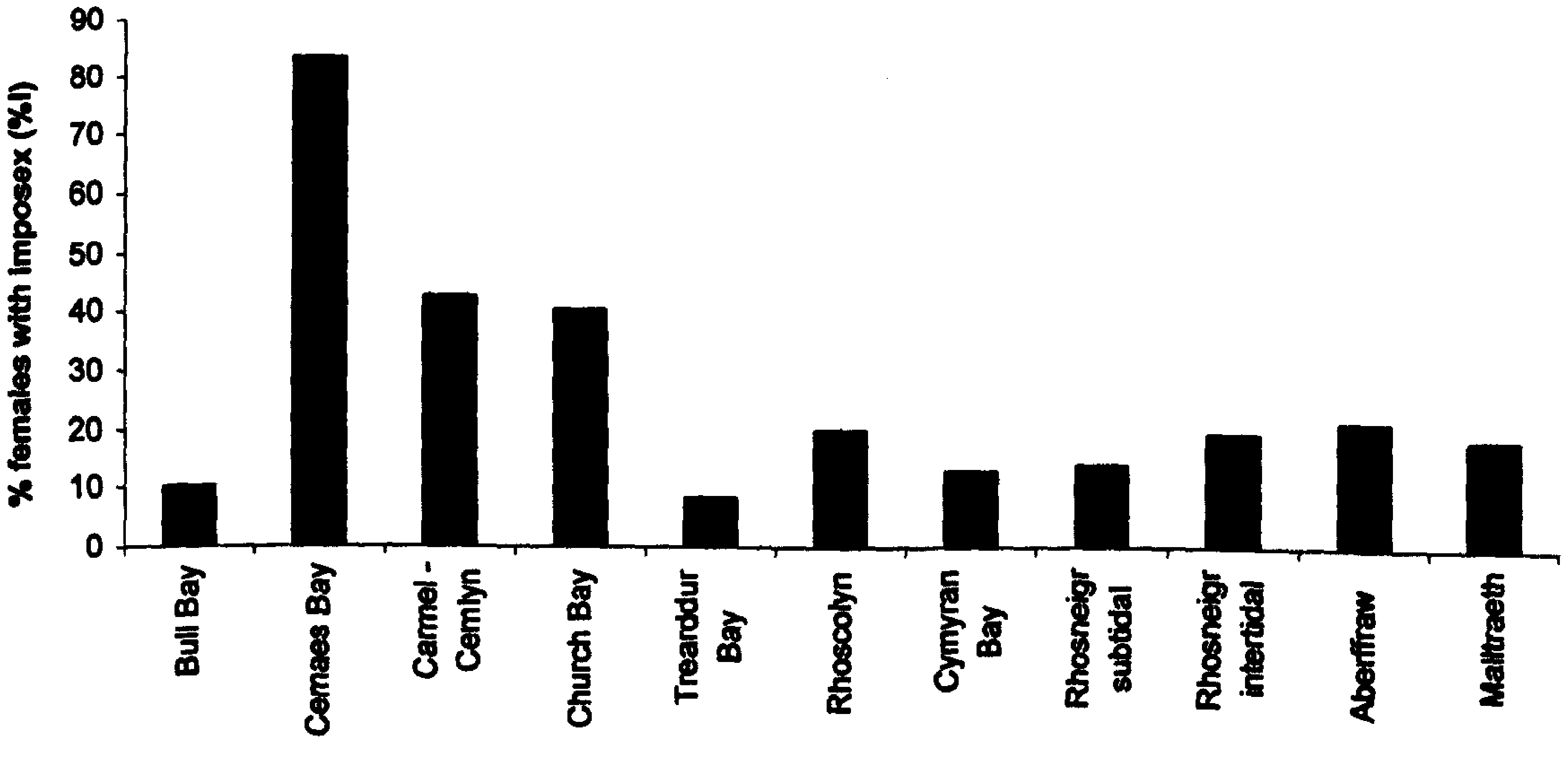


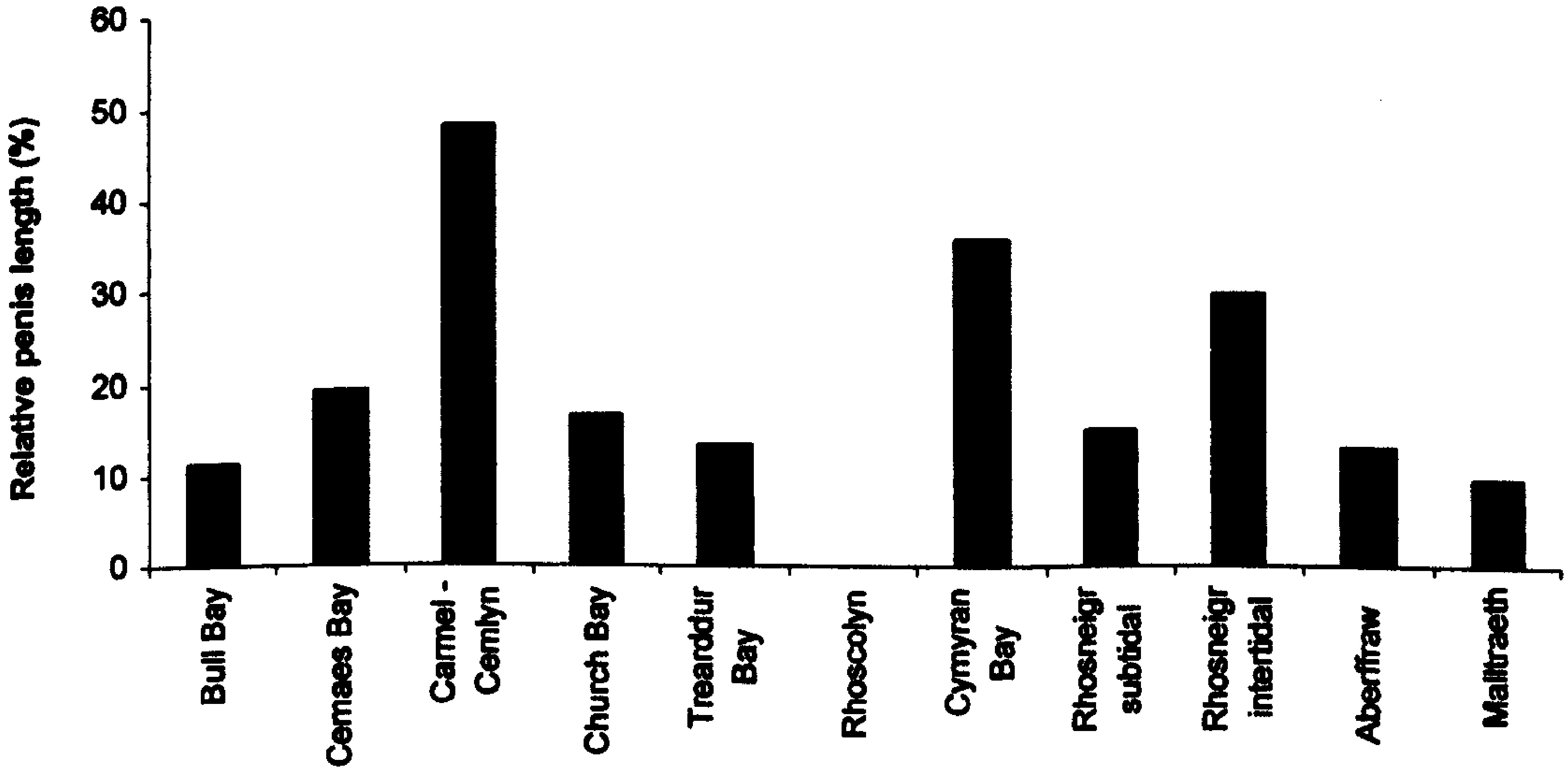
Table 7.3 Results of chi-square analyses (x^2, p) of the percentage of imposex affected *Nassarius reticulatus* females (I%) from 11 sites around Anglesey. Data in bold represent comparisons where there was no significant difference between the sites (p>0.05).

	Cemaes Bay	Carmel - Cemlyn	Church Bay	Trearddur Bay	Rhoscolyn	Cymyran Bay	Rhosneigr subtidal	Rhosneigr intertidal	Aberffraw	Malltraeth
Bull Bay	x^2= 107.1 p<0.001	x^2= 26.6 p<0.001	x^2= 24.0 p<0.001	**x^2= 0.24** **p=0.621**	x^2= 3.92 p=0.048	**x^2= 0.422** **p=0.506**	**x^2= 0.76** **p=0.384**	**x^2= 3.27** **p=0.071**	x^2= 4.62 p=0.032	**x^2= 3.27** **p=0.071**
Cemaes Bay		x^2= 35.861 p<0.001	x^2= 39.05 p<0.001	x^2= 113.42 p<0.001	x^2= 79.45 p<0.001	x^2= 98.16 p<0.001	x^2= 95.31 p<0.001	x^2= 81.95 p<0.001	x^2= 77.0 p<0.001	x^2= 81.95 p<0.001
Carmel - Cemlyn			**x^2= 0.083** **p=0.774**	x^2= 30.83 p<0.001	x^2= 11.31 p=0.001	x^2= 21.09 p<0.001	x^2= 19.44 p<0.001	x^2= 12.48 p<0.001	x^2= 10.22 p=0.001	x^2= 12.48 p<0.001
Church Bay				x^2= 28.07 p<0.001	x^2= 9.52 p=0.002	x^2= 18.71 p<0.001	x^2= 17.15 p<0.001	x^2= 10.60 p=0.001	x^2= 8.52 p=0.004	x^2= 10.60 p=0.001
Trearddur Bay					x^2= 5.98 p=0.014	**x^2= 1.33** **p=0.249**	**x^2= 1.84** **p=0.175**	x^2= 5.18 p=0.023	x^2= 6.82 p=0.009	x^2= 5.18 p=0.023
Rhoscolyn						**x^2= 1.78** **p=0.182**	**x^2= 1.28** **p=0.259**	**x^2= 0.032** **p=0.858**	**x^2= 0.031** **p=0.861**	**x^2= 0.032** **p=0.858**
Cymyran Bay							**x^2= 0.043** **p=0.836**	**x^2= 1.339** **p=0.247**	**x^2= 2.268** **p=0.132**	**x^2= 1.339** **p=0.247**
Rhosneigr subtidal								**x^2= 0.907** **p=0.341**	**x^2= 1.697** **p=0.193**	**x^2= 0.907** **p=0.341**
Rhosneigr intertidal									**x^2= 0.125** **p=0.724**	**x^2= 0** **p=1.000**
Aberffraw										**x^2= 0.125** **p=0.724**
Malltraeth										

The percentage of females affected by imposex was lower at all the sites located on the south-west coast of Anglesey. No ports or marinas are present along the south-west coastal area, although watersport activities are common. The lowest percentage of imposex affected females was observed in Trearddur Bay (8.3%). There was no significant difference regarding the imposex incidence at Rhoscolyn, Cymyran Bay, Rhosneigr subtidal, Rhosneigr intertidal lagoon, Aberffraw and Malltraeth (13-21.3%), which are all located along the west Anglesey coast and are characterised by low to moderate boating activity. The imposex incidence recorded in Bull Bay (10%) was lower than expected, given the level of boating activity there, but may have resulted from the small and probably not representative sample of *Nassarius reticulatus* collected from that area. Bull Bay is located on the north coast of Anglesey and is characterised by moderate to high boating activity. A small marina is also located in Bull Bay.

Relative penis length (RPL) was estimated for all the populations of *Nassarius reticulatus* around Anglesey (table 7.2) and is presented in figure 7.9. The highest RPL was observed in the area of Carmel-Cemlyn (48.2%), where the imposex incidence was also high. The RPL was also high in Cymyran Bay (35.5%) and in the Rhosneigr intertidal lagoon (29.7%), although these areas had a low imposex incidence. The lowest RPL was recorded for the Malltraeth area, where boating activity is low and only 18.6% of females were affected by imposex.

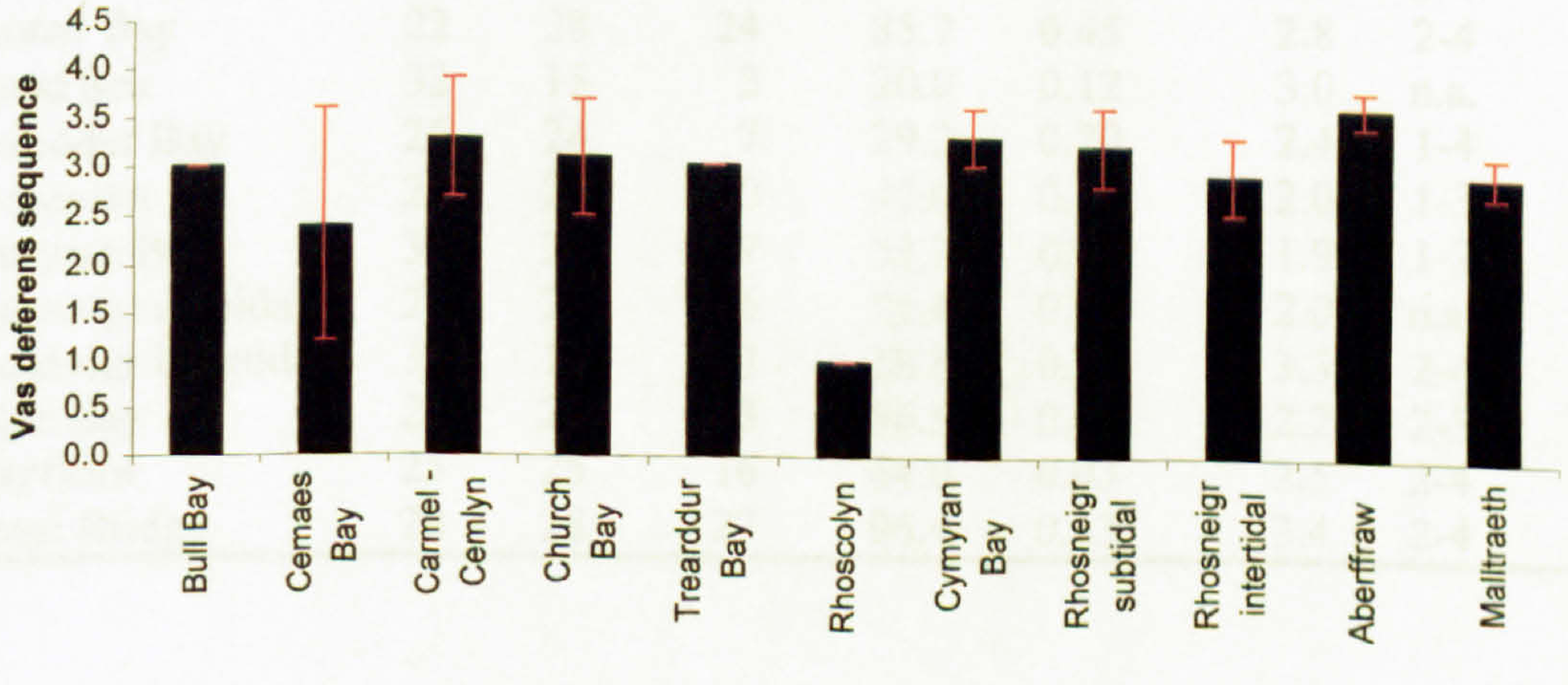
Figure 7.9 Relative penis length (RPL) index for *Nassarius reticulatus* populations from 11 different sites around Anglesey. In the area of Rhoscolyn the one and only female that was affected by imposex was at a VDS stage of 1, where the size of penis was not yet measurable.



A measurable penis was present in imposex affected females from all the Anglesey populations, except for those from Rhoscolyn. Only a small sample of *Nassarius reticulatus* was collected in Rhoscolyn, and only one female showed indications of imposex. The female was at a VDS stage equal to 1 and was characterised by the presence of a tiny, not yet measurable, penis. The absence of a penis is not synonymous with no imposex and the VDS index based on the development of vas deferens allowed a better description of imposex.

Mean vas deferens sequence (VDS) was estimated for all *Nassarius reticulatus* populations around Anglesey (table 7.2; figure 7.10). There was no significant difference regarding the mean VDS between the different sites (Anova: F=1.62, p=0.141), with the exception of Rhoscolyn where only one imposex affected female was found with a VDS stage of 1a. Although the incidence of imposex was higher in some areas (Cemaes Bay, Carmel-Cemlyn and Church Bay) the VDS stage of imposex affected females was similar between low and high imposex affected areas. Most of the *N. reticulatus* examined from the 11 sites exhibited the early stages of imposex with a VDS stage ranging from 1 to 4. No cases of VDS higher than 4 were observed around Anglesey. Only one whelk collected from Carmel to Cemlyn area was classified at the 1b VDS stage (short vas deferens without penis), and this represented only 3.03% of the total females examined from that area. No gastropods with VDS stage 3b were found.

Figure 7.10 Mean vas deferens sequence (VDS) index for the *Nassarius reticulatus* populations from 11 different sites around Anglesey. The error bars represent the 95% confidence intervals of the means (1.96·SE).



No sterile females carrying aborted egg capsules were observed and there was no sign of blockage of the vulva. Only one imposex affected female with VDS equal to 4 had a clearly convoluted oviduct, classified at an AOS stage equal to 2. This female was collected in the area around Carmel Head to Cemlyn Bay, which was characterised by a high incidence of imposex (42.4%) and also by the highest RPL (48.2%).

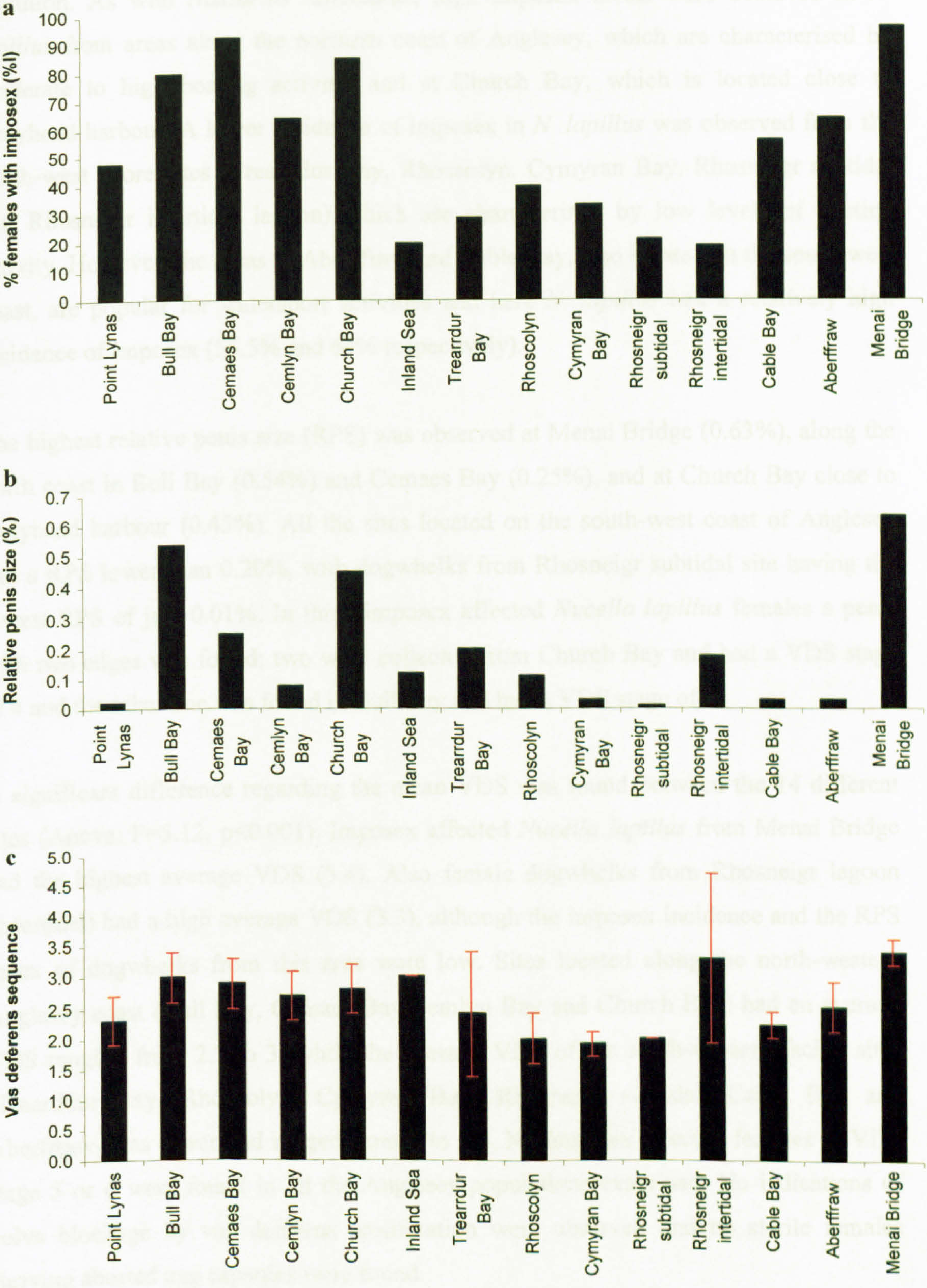
7.3.4 Imposex in *Nucella lapillus*

Levels of imposex in *Nucella lapillus* were examined mostly from sites where *Nassarius reticulatus* was present. Some additional sites (Menai Bridge, Cable Bay, Point Lynas), where *N. reticulatus* was not present, were also examined for *N. lapillus*, in order to compare current imposex indices with previous studies. The imposex incidence (I%), the relative penis size (RPS) and the vas deferens sequence (VDS) of 14 *N. lapillus* populations around Anglesey are shown in table 7.4 and figure 7.11.

Table 7.4 Imposex indices for *Nucella lapillus* from 14 sites around Anglesey. The percentage of females with imposex (I%), the relative penis size (RPS) and the vas deferens sequence (VDS) (mean, range and standard error) are presented for each site. The numbers of males, females and imposex affected females are also indicated. n.a.: not-applicable, when all dogwhelks were at the same VDS stage.

Sites	Number of			I %	RPL %	VDS		
	♂	♀	imposex			mean	range	SE
Point Lynas	27	23	11	47.8	0.02	2.3	1-4	0.2
Bull Bay	24	30	24	80.0	0.54	3.0	1-4	0.2
Cemaes Bay	23	27	25	92.6	0.25	2.9	1-4	0.2
Cemlyn Bay	19	31	20	64.5	0.08	2.7	2-4	0.2
Church Bay	22	28	24	85.7	0.45	2.8	2-4	0.2
Inland Sea	32	15	3	20.0	0.12	3.0	n.a.	n.a.
Trearddur Bay	26	24	7	29.2	0.20	2.4	1-4	0.5
Rhoscolyn	25	25	10	40.0	0.11	2.0	1-3	0.2
Cymyran Bay	32	21	7	33.3	0.03	1.9	1-2	0.1
Rhosneigr subtidal	22	28	6	21.4	0.01	2.0	n.a.	n.a.
Rhosneigr intertidal	32	16	3	18.8	0.17	3.3	2-4	0.7
Cable Bay	27	23	13	56.5	0.03	2.2	2-3	0.1
Aberffraw	25	25	16	64.0	0.03	2.5	2-4	0.2
Menai Bridge	20	28	27	96.4	0.63	3.4	2-4	0.1

Figure 7.11 Imposex indices of *Nucella lapillus* from 14 sites around Anglesey. a) percentage of females affected by imposex (I%), b) relative penis size (RPS) and c) mean vas deferens sequence (VDS) ± 95% confidence intervals (1.96·SE).



A chi-square analysis comparing the percentage of imposex affected females from the different sites is shown in table 7.5. The highest imposex incidence for *Nucella lapillus* was found at Menai Bridge (96.4%) and Cemaes Bay (92.6%), while Church Bay (85.7%), Bull Bay (80%) and Cemlyn Bay (64.5%) were also highly affected by TBT pollution. As with *Nassarius reticulatus*, high imposex levels were observed in *N. lapillus* from areas along the northern coast of Anglesey, which are characterised by moderate to high boating activity, and at Church Bay, which is located close to Holyhead harbour. A lower incidence of imposex in *N. lapillus* was observed from the south-west shore sites (Trearddur Bay, Rhoscolyn, Cymyran Bay, Rhosneigr subtidal and Rhosneigr intertidal lagoon) which are characterised by low levels of boating activity. However, the areas of Aberffraw and Cable Bay, also located on the south-west coast, are popular for watersport activities and here *N. lapillus* had a relatively high incidence of imposex (56.5% and 64% respectively).

The highest relative penis size (RPS) was observed at Menai Bridge (0.63%), along the north coast in Bull Bay (0.54%) and Cemaes Bay (0.25%), and at Church Bay close to Holyhead harbour (0.45%). All the sites located on the south-west coast of Anglesey had a RPS lower than 0.20%, with dogwhelks from Rhosneigr subtidal site having the lowest RPS of just 0.01%. In three imposex affected *Nucella lapillus* females a penis with two edges was found; two were collected from Church Bay and had a VDS stage of 4 and the other one was found in Bull Bay and had a VDS stage of 3.

A significant difference regarding the mean VDS was found between the 14 different sites (Anova: F=5.12, $p<0.001$). Imposex affected *Nucella lapillus* from Menai Bridge had the highest average VDS (3.4). Also female dogwhelks from Rhosneigr lagoon (intertidal) had a high average VDS (3.3), although the imposex incidence and the RPS index of dogwhelks from this area were low. Sites located along the north-western Anglesey coast (Bull Bay, Cemaes Bay/Cemlyn Bay and Church Bay) had an average VDS ranging from 2.7 to 3, while the average VDS of the south-western facing sites (Trearddur Bay, Rhoscolyn, Cymyran Bay, Rhosneigr subtidal, Cable Bay and Aberffraw) was lower and ranged from 1 to 2.5. No imposex affected females of VDS stage 5 or 6 were found in all the Anglesey populations examined. No indications of vulva blockage by vas deferens proliferation were observed and no sterile females carrying aborted egg capsules were found.

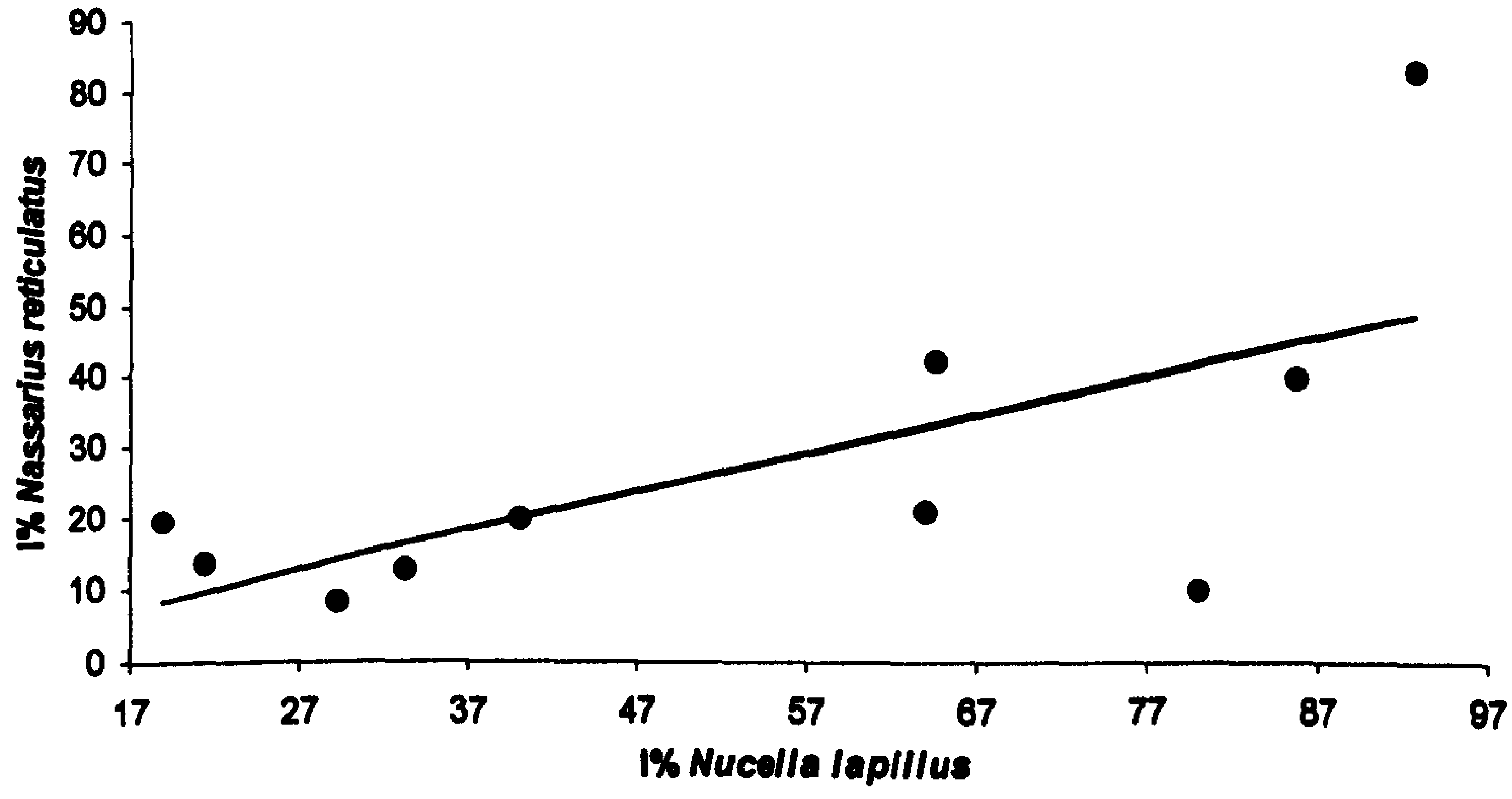
Table 7.5 Results of chi-square analyses (x^2, p) for the percentage of imposex affected *Nucella lapillus* females (I%) collected from 14 sites around Anglesey. Data in bold represent comparisons between sites which are not significantly different ($p>0.05$).

	Bull Bay	Cemaes Bay	Cemlyn Bay	Church Bay	Inland Sea	Trearddur Bay	Rhoscolyn	Cymyran Bay	Rhosneigr subtidal	Rhosneigr intertidal	Cable Bay	Aberffraw	Menai Bridge
Point Lynas	22.20 <0.001	48.68 <0.001	5.46 0.019	32.65 <0.001	17.47 <0.001	7.62 0.006	**1.30** **0.254**	4.669 0.031	16.13 <0.001	18.88 <0.001	**1.43** **0.231**	5.20 0.023	57.14 <0.001
Bull Bay		7.24 0.007	6.12 0.013	**1.28** **0.259**	72.0 <0.001	52.45 <0.001	33.3 <0.001	44.94 <0.001	69.63 <0.001	74.43 <0.001	12.86 <0.001	6.35 0.012	12.12 <0.001
Cemaes Bay			24.52 <0.001	**2.61** **0.106**	108.41 <0.001	86.09 <0.001	63.05 <0.001	72.22 <0.001	105.75 <0.001	111.12 <0.001	35.48 <0.001	24.92 <0.001	**0.87** **0.352**
Cemlyn Bay				12.60 <0.001	40.51 <0.001	25.23 <0.001	11.95 0.001	19.78 <0.001	38.59 <0.001	42.49 <0.001	**1.33** **0.250**	**0.003** **0.958**	31.57 <0.001
Church Bay					87.44 <0.001	66.48 <0.001	45.39 <0.001	58.28 <0.001	84.92 <0.001	90.01 <0.001	21.39 <0.001	12.91 <0.001	6.11 0.013
Inland Sea						**2.19** **0.139**	9.52 0.002	4.34 0.037	**0.03** **0.861**	**0.03** **0.858**	28.23 <0.001	39.74 <0.001	118.56 <0.001
Trearddur Bay							**2.68** **0.102**	**0.37** **0.541**	**1.71** **0.191**	**2.74** **0.098**	15.45 <0.001	24.62 <0.001	95.77 <0.001
Rhoscolyn								**1.06** **0.304**	8.52 0.004	10.60 0.001	5.44 0.020	11.54 0.001	72.06 <0.001
Cymyran Bay									**3.65** **0.056**	5.09 0.024	11.16 0.001	19.24 <0.001	86.67 <0.001
Rhosneigr subtidal										**0.13** **0.724**	26.57 <0.001	37.83 <0.001	115.85 <0.001
Rhosneigr intertidal											29.95 <0.001	41.71 <0.001	121.31 <0.001
Cable Bay												**1.2** **0.273**	43.27 <0.001
Aberffraw													32.0 <0.001
Malltraeth													

7.3.5 Comparison between *Nassarius reticulatus* and *Nucella lapillus* as imposex indicators

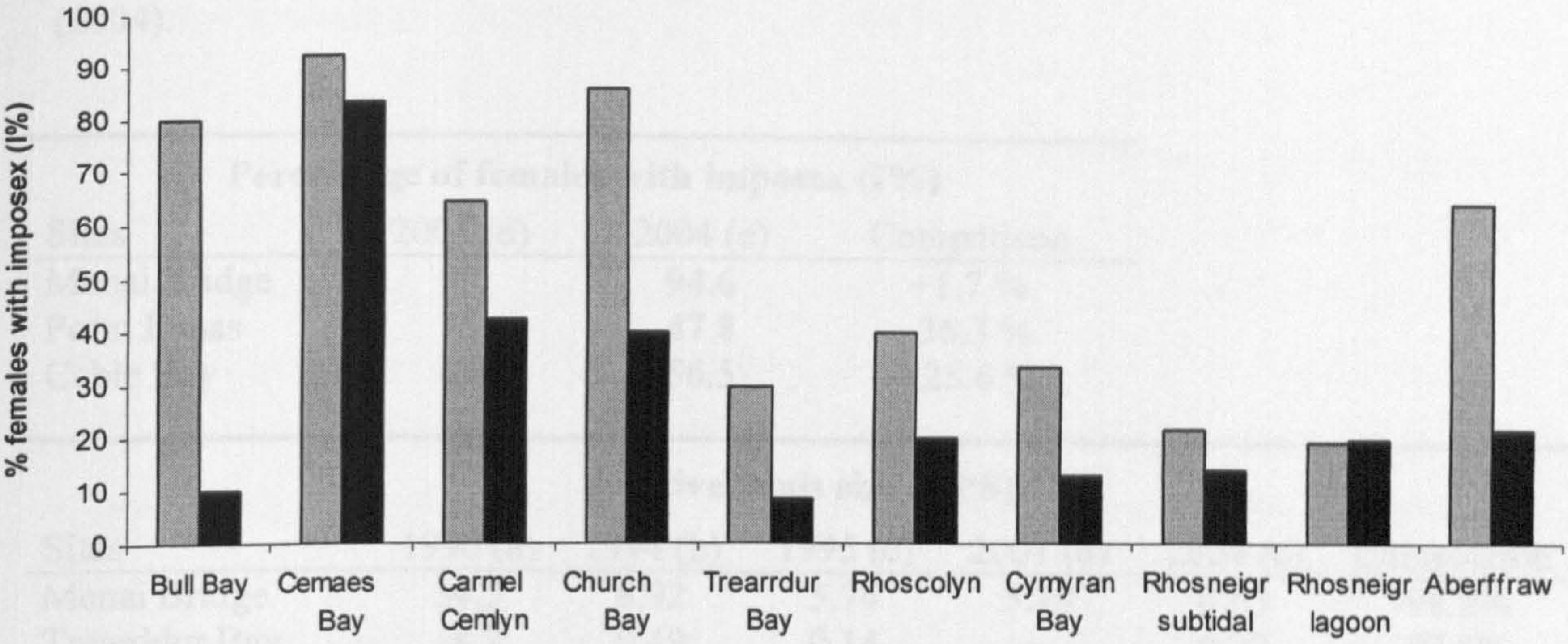
The imposex indices I%, RPL and VDS of sympatric populations of *Nassarius reticulatus* and *Nucella lapillus* were compared in order to evaluate the TBT impact on the two different species. A significant correlation (r=0.666, p=0.036) was found between the percentage of imposex affected *N. reticulatus* and *N. lapillus* females from their respective Anglesey sites. A high imposex incidence (I%) for *N. reticulatus* was observed in the same sites where higher imposex levels were also found in *N. lapillus*. A significant regression (F=6.37, p=0.036, normal data A^2=0.346, p=0.403) was observed for *N. lapillus* and *N. reticulatus* imposex indices (figure 7.12). However, the mean length of the female penis (LFP) and the imposex indices RPL/RPS and VDS were not significantly correlated for the two species (for LFP: r = -0.014, p=0.972; for RPL/PRS: r =0.408, p=0.276; for VDS: r =0.176, p=0.626).

Figure 7.12 Correlation of the percentage of female *Nassarius reticulatus* and *Nucella lapillus* affected by imposex (I%). The regression is described by the equation:
N. reticulatus I% = -2 +0.551 ·*N. lapillus* I%.



In most sites where imposex was examined, *Nucella lapillus* exhibited a higher imposex incidence than *Nassarius reticulatus* (figure 7.13), indicating that *N. lapillus* is more seriously affected and more sensitive to TBT pollution.

Figure 7.13 Comparison of the percentage of imposex affected females (%I) between *Nassarius reticulatus* (black bars) and *Nucella lapillus* (grey bars).



7.3.6 Recovery of imposex affected populations of *Nucella lapillus*

The levels of imposex in *Nucella lapillus* from around Anglesey, obtained in the present study (2004), are compared with levels obtained in previous studies (table 7.6). The percentage of imposex affected *N. lapillus* at Menai Bridge in 2004 had slightly increased (+1.7%) in comparison with the imposex incidence recorded in 2001. Fewer females were affected by imposex at Point Lynas in 2004 (-36.3%), whilst an increase in imposex incidence was observed at Cable Bay (+25.6%) during the last 3 years. A dramatic decrease in the RPS index was observed during the last 14 years at Menai Bridge, Trearddur Bay, Rhosneigr, Church Bay, Bull Bay and during the last 3 years at Point Lynas and Cable Bay. The average VDS index for most populations was also notably decreased within the 14-year period. However, the average VDS had increased during the last 3 years at Point Lynas (6%) and Cable Bay (41.9%).

Table 7.6 Comparison of imposex indices in mature *Nucella lapillus* (I%, RPS and VDS) collected between 1990 and 2004 from sites around Anglesey. The percentage increase (+) or decrease (-) from the oldest study until present is indicated. (a) Meredith (1990), (b) Dukes (1994), (c) Wagiman (1995), (d) Saurel (2002), (e) present study (2004).

Percentage of females with imposex (I%)

Sites	2001 (d)	2004 (e)	Comparison
Menai Bridge	93	94.6	+1.7 %
Point Lynas	75	47.8	-36.3 %
Cable Bay	45	56.5	+25.6 %

Relative penis size (RPS)

Sites	1990 (a)	1994 (b)	1995 (c)	2001 (d)	2004 (e)	Comparison
Menai Bridge	34.5	8.92	5.74	5.19	0.63	-98.2%
Trearddur Bay	8.2	0.49	0.14	-	0.20	-97.6%
Rhosneigr subtidal	8.7	-	-	-	0.01	-99.9%
Church Bay	4.8	-	-	-	0.45	-90.6%
Bull Bay	11.0	-	-	-	0.54	-95.1%
Point Lynas	-	-	-	0.18	0.02	-88.9%
Cable Bay	-	-	-	0.39	0.03	-92.3%

Vas Deferens Sequence (VDS)

Sites	1990 (a)	1994 (b)	1995 (c)	2001 (d)	2004 (e)	Comparison
Menai Bridge	5.23	3.45	3.63	3.38	3.4	-35.0%
Trearddur Bay	4.06	3.50	2.17	-	2.4	-40.9%
Rhosneigr subtidal	4.08	-	-	-	2.0	-51.0%
Church Bay	3.47	-	-	-	2.8	-19.3%
Bull Bay	4.06	-	-	-	3.0	-26.1%
Point Lynas	-	-	-	2.17	2.3	+6.0%
Cable Bay	-	-	-	1.55	2.2	+41.9%

7.4 DISCUSSION

A predominance of females is quite common in gonochoristic molluscs (Fretter & Graham, 1994). A high percentage of female *Nassarius reticulatus* were present at all the Anglesey sites examined. The percentage of females ranged from 60% to 91.2%, and the percentage of males from 8.8% to 40%. The highest percentage of females was observed in the intertidal lagoon at Rhosneigr. The sex ratio was similar in most of the sites examined, with an average of 73.5% females and 26.5% males (sex ratio of 3:1). A higher female to male sex ratio was observed during the present study than has been reported elsewhere in previous studies. Stroben *et al.* (1992b) indicated that a population of *N. reticulatus* in France consisted of 56.3% females and 43.7% males, and similarly Pelseneer (1926) recorded a frequency of 60% females and 40% males. Tallmark (1980) observed a sex ratio of 52% males and 48% females in a *N. reticulatus* population in Gullmar fjord (Sweden).

High imposex levels may be responsible for a reduction in the proportion of females in a population due to increased mortality (Feare, 1970a), as has been observed in *Nucella lapillus* and *Ilyanassa obsoleta* (Bryan *et al.*, 1986). Gibbs *et al.* (1988) indicated that when TBT pollution is applied early enough in the life history of *N. lapillus* and at sufficient strength, a complete sex reversal can be caused, where oogenesis is replaced by spermatogenesis. However, Bryan *et al.* (1993) has indicated that the sex ratio of *Nassarius reticulatus* is not affected by TBT pollution. The percentage of *N. reticulatus* females in SW England populations before the TBT restriction in 1987 ranged from 47% to 90%, while 5-6 years after the introduction of legislation it ranged from 43% to 60% (Bryan *et al.*, 1993). Similarly, in the present study there were no significant differences in the sex ratio between high and low imposex affected populations.

The average penis size of male *Nassarius reticulatus* in France was 13.0 mm and the average female penis was 2.0 mm (Stroben *et al.*, 1992b), while in west Portugal the mean male penis was 12.1 mm and the mean female penis length varied between 0 and 10.7 mm (Barroso *et al.*, 2002). Barreiro *et al.* (2001) observed that in NW Spain the average female penis ranged from 2.2 to 10.3 mm, while the average male penis from 8.8 to 12.7 mm. The male penis length of *N. reticulatus* in populations studied in SW England ranged from 8.1 to 12 mm, and the female penis ranged from 0.2 to 7.1 mm (Bryan *et al.*, 1993). The size of the male penis is related to body size which varies considerably between different sites (Gibbs & Bryan, 1994). During the current study the penis of *N. reticulatus* males had a size range from 2.1 to 15.0 mm, while the penis of imposex affected females ranged from 0.1 to 6.6 mm. Penis size in males and imposex-affected females increases exponentially with shell length. However, penis growth was different between the two genders. A measurable penis was present in imposex affected females from almost all the Anglesey populations examined. At Rhoscolyn, where only a small sample (N=8) of *N. reticulatus* could be collected, only one female showed indications of imposex. This female had a VDS stage of 1 and was characterised by the presence of a tiny penis.

The highest imposex incidence (83.3%) was observed in *Nassarius reticulatus* from Cemaes Bay, located on the north coast of Anglesey, where there is moderate to high boating activity. A small port, where several fishing and leisure boats are anchored is present in this site. A relatively high imposex incidence (42.4%) and the highest RPL (48.2%) were also observed in whelks from the nearby area of Carmel Head-Cemlyn Bay, which is characterised by moderate boating activity. A high percentage of imposex affected females was also found at Church Bay (40%), which is located on the north-west coast of Anglesey. Although Church Bay has a low level of boating activity, TBT polluted waters from the Holyhead port located adjacent and in close proximity to the bay, may be responsible for the increased imposex incidence observed. The north-east direction of the residual currents (see chapter 2, figure 2.4) along the coast of Anglesey may transfer TBT-polluted water masses from Holyhead port to the north-western and northern coastal areas around Anglesey. Bull Bay on the north coast of Anglesey has a small marina and is characterised by moderate to high boating activity. However, the incidence of imposex at Bull Bay was lower than expected (10%), probably due to the small number of *N. reticulatus* that were found in the area. The percentage of females affected with imposex was less than 21.3% for all the sites located along the south-west coast of Anglesey, where no ports or marinas are present and where boating activity is at a moderate to low level. The lowest percentage of imposex affected females was observed in Trearddur Bay (8.3%). The lowest RPL (9.2%) was recorded in Malltraeth, where boating activity was low and only 18.6% of females were affected by imposex. The RPL was increased in *N. reticulatus* from Cymyran Bay (35.5%) and Rhosneigr intertidal lagoon (29.7%), although these areas had a low imposex incidence. According to Stroben *et al.* (1992a) female *N. reticulatus* develop obvious imposex characters even in areas where TBT exposure is low.

The mean VDS index ranged from 2.4 to 3.6 and there was no significant difference between the populations, with the exception of Rhoscolyn (only 1 imposex affected female at VDS =1). The VDS stages of type b (1b, 3b) were not common. Only 3.03% of the females examined from the area Carmel Head to Cemlyn Bay was classified at the 1b VDS stage and no whelks in 3b stage were found. Stroben *et al.* (1992b) also found that stages 1b and 3b occurred only in a very small percentage (1.09% and 0.17% respectively) of an imposex affected *Nassarius reticulatus* population in France. Barroso *et al.* (2002) indicated that only 2.4% of the imposex affected females from the west coast of Portugal presented VDS stages 1b and 3b. No imposex affected females of VDS stage 4+, where the vas deferens joins or runs parallel to the capsule gland, were found in Anglesey during the present study. Stroben *et al.* (1992b) reported that only 3.09% of an imposex affected *N. reticulatus* population in France had a VDS of 4+. Also Barreiro *et al.* (2001) and Barroso *et al.* (2002) reported the presence of females with a 4+ VDS stage in Spain and Portugal respectively.

Initial studies indicated that imposex pollution did not cause restrictions of fertility in imposex affected populations of *Nassarius reticulatus*, and the histological structure of the ovary remained normal (Stroben *et al.*, 1992a; 1992b). Stroben *et al.* (1992b) indicated that in *N. reticulatus* the vaginal opening was unmodified and the capability for copulation was retained, thus deposition of normal egg capsules could occur. However, later studies revealed the occurrence of sterile female *N. reticulatus* in Spain (Barreiro *et al.*, 2001) and Portugal (Barroso *et al.*, 2002). The aborted capsules formed firm irregular masses, easily distinguishable through the capsule gland walls (Barreiro *et al.*, 2001). The frequency of sterile females varied between 5 to 50% of the total females per site in Portugal (Barroso *et al.*, 2002), and sterility occurred only in females highly affected with imposex (mostly VDS 4+) (Barreiro *et al.*, 2001; Barroso *et al.*, 2002). No sterile females carrying aborted egg capsules were observed in the Anglesey populations, and there were no signs of blockage of the vulva by vas deferens proliferation, as reported for *Nucella lapillus* by Gibbs *et al.* (1987) or by any kind of smooth or solid cylindrical tissue as reported for *N. reticulatus* by Barroso *et al.* (2002).

A clearly convoluted oviduct (AOS=2) was observed only in one highly imposex affected female (VDS = 4) from the area around Carmel Head and Cemlyn Bay, which was characterised by a high imposex incidence (42.4%) and also by the highest RPL (48.2%). Stroben *et al.* (1992b), Barroso *et al.* (2002) and Barreiro *et al.* (2001) reported the presence of a coiled oviduct in *Nassarius reticulatus* populations in France, Portugal and Spain, respectively. Barroso *et al.* (2002) indicated that oviduct convolution occurred only in females with a VDS stage of 3 or higher. Half of the females that exhibited VDS stage 4+ in western Portugal had a convoluted oviduct (Barroso *et al.*, 2002). Barreiro *et al.* (2001) indicated that from the total number of *N. reticulatus* females examined in Spain, 19.6% presented a slightly sinuous oviduct (AOS=1) and 6.9% a convoluted oviduct (AOS=2). Masculinisation of the gonadial oviduct was mostly limited to females presenting VDS stages of 4 or 4+ (Barreiro *et al.*, 2001).

Barreiro *et al.* (2001) indicated that *N. reticulatus* collected from areas linked to commercial harbours in SW Spain had an RPL index which exceeded 80% and the VDS index reached the maximum of 4.5 with a narrow range from 4 to 4+ stage. By contrast, samples from areas with little or no commercial shipping were characterised by an RPL below 60% (17-56%) and a VDS index below 4 (2.3-3.7), with a wider range from 1 to 4+ (Barreiro *et al.*, 2001). *N. reticulatus* collected some distance away from commercial ports in SW England had an RPL index lower than 59% (usually 10 to 35%) under post-ban conditions (Bryan *et al.*, 1993). However, Barreiro *et al.* (2001) indicated that female *N. reticulatus* collected in Spain from stations with small marinas sheltering yachts and small boats year-round, were also highly affected by imposex (RPL 88.5%, VDS 4 to 4+), although they were not close to large commercial harbours. Similarly, during the present study, *N. reticulatus* that were collected around Anglesey from the sampling stations close to large commercial harbours (i.e. Church Bay) or small marinas (i.e. Cemaes Bay, Carmel Head-Cemlyn Bay) exhibited higher imposex indices.

In conclusion, the percentage of imposex affected female *Nassarius reticulatus* from around the coastal waters in Anglesey ranged from 8.3% to 83.3% and the RPL index ranged from 9.2% to 48.2%. The mean VDS stage of all populations examined was similar (2.4-3.6) and no imposex affected females with a VDS stage of 4+ were found. Also no sterile females were recorded from Anglesey. Only one female with a clearly convoluted oviduct that was highly affected by imposex (VDS 4) was collected from the area of Carmel Head-Cemlyn Bay. In previous studies the imposex incidence of *N. reticulatus* in France was 83.75% (Stroben *et al.*, 1992b) and on the west coast of Portugal it ranged between 0 and 100% (Barroso *et al.*, 2002). Estimates of RPL index ranged between 60-80% in France during 1988-1991 (Stroben *et al.*, 1992b). The RPL index in SW England ranged between 10.2% and 67.2% about 5-6 years after the TBT restrictions were applied in 1987, while before the restrictions the RPL ranged from 28.9% to 82.3% (Bryan *et al.*, 1993). The RPL index of *N. reticulatus* in west Portugal ranged between 0 and 92.1% (Barroso *et al.*, 2002) and in north-west Spain from 17.4% to 93.7% (Barreiro *et al.*, 2001). Imposex affected females of VDS stage 4+, as well as females with a convoluted oviduct, were found in France (Stroben *et al.*, 1992b), Portugal (Barroso *et al.*, 2002) and Spain (Barreiro *et al.*, 2001). Also sterile females were recorded in Portugal (Barroso *et al.*, 2002) and Spain (Barreiro *et al.*, 2001). The *N. reticulatus* populations around Anglesey in 2004 therefore appear to be less affected by TBT pollution compared with populations in France (Stroben *et al.*, 1992b), Portugal (Barroso *et al.*, 2002), Spain (Barreiro *et al.*, 2001) and SW England (Bryan *et al.*, 1993) which had been examined in previous years.

The imposex indices of sympatric populations of *Nucella lapillus* and *Nassarius reticulatus* were examined in order to compare sensitivity of the two indicator species. The imposex incidence in *N. lapillus* displayed a similar pattern to *N. reticulatus.* A high percentage of imposex affected female *N. lapillus* was similarly found in Cemaes Bay (92.6%), Church Bay (85.7%) and Cemlyn Bay (64.5%). The imposex incidence of *N. lapillus* was high in Bull Bay (80%), as was expected due to the presence of a small marina in the area. Also a high relative penis size (RPS) was observed in *N. lapillus* collected from Bull Bay (0.54%), Cemaes Bay (0.25%) and Church Bay (0.45%), whilst at sites along the west Anglesey coast the RPS was lower than 0.20%. Sites located along the north and north-west Anglesey coast (Bull Bay, Cemaes Bay, Cemlyn Bay and Church Bay) had an average VDS that ranged between 2.7 and 3, while the average VDS of the south-west Anglesey populations was lower and ranged between 1 and 2.5. As with *N. reticulatus*, the populations of *N. lapillus* were highly affected by imposex along the northern coast of Anglesey, where there is moderate to high boating activity, and in Church Bay, which is located close to Holyhead port. Lower imposex incidence was observed at the south-western sites, characterised by low boating activity.

Interspecific comparisons between sympatrical populations of different imposex indicator species have been conducted by Stroben *et al.* (1992a) and Bryan *et al.* (1993) for *Nassarius reticulatus* and *Nucella lapillus* and by Oehlmann *et al.* (1998) for *Hinia incrassata* and *N. lapillus* and for *H. incrassata* and *N. reticulatus.* Stroben *et al.* (1992a) indicated that there was a significant exponential relationship between the imposex indices RPL, VDS and the average female penis length of *N. reticulatus* and *N. lapillus.* According to Stroben *et al.* (1992a) imposex development in both species was comparable. During the present study there was no significant correlation between the female penis length of *N. reticulatus* and *N. lapillus*, between the RPL index of *N. reticulatus* and the RPS index of *N. lapillus*, or between the VDS indices of the two species. The small samples of *N. reticulatus* that were collected from some areas could be responsible for the increased variability of the above imposex indices. However, the significant correlation observed between *N. reticulatus* and *N. lapillus* imposex incidence (I %) indicated that increased percentages of imposex affected females occurred at the same sites for both species.

In most of the sites where imposex was examined for both species, *Nucella lapillus* exhibited a higher incidence than *Nassarius reticulatus*, indicating that *N. lapillus* is more severely affected and therefore more sensitive to TBT pollution. For example, *N. reticulatus* from Aberffraw displayed a low incidence (21.3%), while *N. lapillus* from the same area and nearby Cable Bay displayed a relatively high incidence (56.5% and 64% respectively). *N. lapillus* has been documented as a very sensitive species to TBT pollution by several investigators (Stroben *et al.*, 1992a; 1992b; Bryan *et al.*, 1993). According to Barroso *et al.* (2000) the differences in imposex levels between *N. reticulatus* and *N. lapillus* are related to the differences in habitat type and diet type between the two species. *N. reticulatus* accumulates only 36.9% of the *N. lapillus* TBT-Sn body burden under the same exposure conditions (Stroben *et al.*, 1992a). Also Bryan *et al.* (1993) indicated that *N. reticulatus* degraded TBT more efficiently than *N. lapillus*.

The fact that no imposex affected females of VDS stage 5 or 6 were found in all the *Nucella lapillus* populations examined around Anglesey indicated that TBT pollution in relatively low along the Anglesey coast. Also no indications of vulva blockage by vas deferens proliferation were observed and no sterile females with aborted egg capsules were found around Anglesey. However, a bifurcated penis was observed in three *N. lapillus* females that were collected from areas with high TBT pollution. Two of them were collected in Church Bay (VDS = 4), whilst the other was found in Bull Bay (VDS =3). Oehlmann *et al.* (1991) have noted the presence of two penes in *N. lapillus* imposex affected females. A similar reference, regarding the formation of a bifurcate penis or a double penis in *Nassarius reticulatus* has been reported by Stroben *et al.* (1992b).

A major advantage of using *Nassarius reticulatus* as an imposex indicator species is its wider geographical distribution compared to *Nucella lapillus* (Stroben *et al.*, 1992a). *N. reticulatus* can be found from the Azores and Canaries, north to Norway and throughout the Mediterranean and the Black Sea (Fretter & Graham, 1984). By contrast, *N. lapillus* is completely absent from Belgium, Netherlands, Germany (with the exception of Heligoland), Denmark and the Baltic Sea (Fretter & Graham, 1984). By the early 1990s

N. lapillus populations were declining or were already absent from heavily polluted areas in southern England, France and Norway (Stroben *et al.*, 1992a). By contrast *N. reticulatus* is able to recolonise polluted areas due to the higher dispersion ability of its planktonic veliger larvae (Stroben *et al.*, 1992a). An advantage of using *N. lapillus* as a bioindicator species is the existence of an extensive database regarding imposex development and TBT ecotoxicology (Stroben *et al.*, 1992a). Stroben *et al.* (1992a) proposed that both *N. reticulatus* and *N. lapillus* should be used as TBT bioindicators species when both are found sympatrically. The analysis of more than one TBT-indicator species in biomonitoring programs would result in a broader database on the effects of TBT pollution (Stroben *et al.*, 1992a).

The imposex indices of *Nucella lapillus* recorded during the present study were compared with the results of earlier studies that had been conducted at the same stations around Anglesey. Although the RPS index had decreased by 92.3% in Cable Bay during the last 3 years, the percentage of imposex affected *N. lapillus* had increased by 25.6% and the average VDS was also 41.9% higher within the same time period. However, at most sites an improvement in the imposex indices was recorded. Although the percentage of imposex affected dogwhelks remained almost the same at Menai Bridge (+1.7%) during the last 3 years, the RPS and VDS indices had noticeably decreased by 98.2% and 35% respectively within a period of 14 years after TBT restrictions were applied. Accordingly, the imposex incidence and the RPL index at Point Lynas were 36.3% and 88.9% lower than 3 years ago respectively, while the VDS index was slightly higher (6%). A considerable decrease in the *N. lapillus* RPS and VDS indices was observed during the last 14 years at Trearddur Bay, Rhosneigr, Church Bay and Bull Bay (90.6-99.9% and 19.3-51%, respectively).

Although TBT concentrations in seawater and *Nassarius reticulatus* tissues declined 5 to 10 times in some of the most polluted sites in SW England after the use of TBT was restricted in 1987, the percentage of imposex affected females in *N. reticulatus* populations declined very slowly (Bryan *et al.*, 1993). This was attributed to the longevity of the species, the very slow decline, if any, of the penis length in older females and the limited recruitment of less-affected females (Bryan *et al.*, 1993). Even after the decline of TBT in seawater, exposure of juveniles to polluted sediment or food, where TBT degradation is slower than in the seawater, enhanced the occurrence of imposex (Bryan *et al.*, 1993). Organotin pollution remains an issue of concern around the British coasts. Although large commercial ports appear to be important sources of TBT, areas where there is no commercial shipping still apparently contain numerous imposex affected populations (Barreiro *et al.*, 2001). TBT pollution from commercial shipping may affect areas not immediately adjacent to major ports (Barreiro *et al.*, 2001). Waters leaving highly contaminated areas may carry high levels of TBT for great distances (Barreiro *et al.*, 2001). In addition, fishing vessels larger than 25 m, which are employed in coastal fishing or aquaculture activities, are currently excluded from the EU restrictions on the use of TBT paints, and could be an important source of TBT pollution in coastal waters (Barreiro *et al.*, 2001). The implementation in 1987 of limited restrictions on the use of TBT antifouling paints does not appear to be sufficiently effective, thus regulations for further restrictions have now been considered.

In October 2001 the International Maritime Organisation adopted a new policy regarding TBT anti-fouling paints and a global prohibition on the application of organotin compounds which act as biocides in anti-fouling systems on ships was agreed. By January 1, 2003 all ships cannot apply or re-apply organotin compounds which act as biocides in antifouling systems. Finally, by January 1, 2008 ships will either have to remove such compounds from their hulls or external parts or surfaces, or they will require a coating that prevents such compounds leaching from the underling non-compliant anti-fouling systems (source: http://www.imo.org/home.asp).

In the present chapter the levels of imposex in *Nassarius reticulatus* populations from around Anglesey (North Wales, UK) were examined. Whelks collected from areas that were associated with high boating activity or linked with possible sources of TBT pollution, such as small marinas or Holyhead harbour, exhibited a higher degree of imposex. The sex ratio of imposex affected populations was not affected by TBT pollution. No sterile *N. reticulatus* carrying aborted egg capsules were observed during the present study. The populations of *N. reticulatus* around Anglesey are less affected by TBT pollution compared with populations in SW England, France, Spain or Portugal that were examined several years ago. Although sympatric populations of *Nucella lapillus* were more sensitive to TBT pollution than *N. reticulatus*, both bioindicator species presented a higher degree of imposex in the same areas. A significant recovery of imposex affected populations of *N. lapillus* around Anglesey was recorded over the last 14 years.

CHAPTER 8

General Discussion

The distribution of *Nassarius reticulatus* around Anglesey (North Wales, UK) was examined during July 2003. *N. reticulatus* occur in clumped aggregations in areas where carrion sources are abundant, and immediately after feeding they bury in the sediment in order to decrease predation risks. Whelks living inside aggregations are less susceptible to predation than individuals living outside aggregations (Stoner & Lally, 1994; Ray & Stoner, 1995). The abundance of *N. reticulatus* was maximal at depths of 9-13 m, whereas in shallow (<4m) and depths >17m very few individuals were present. The winter freezing temperatures in shallow areas can cause mortalities and eliminate the populations of *N. reticulatus* (Lambeck, 1982). In the current study smaller whelks were found at depths between 6 and 10 m, suggesting the presence of nursery grounds in this depth range. According to Tallmark (1980) newly settled juvenile *N. reticulatus* spend their first 3 years in nursery grounds, which are rich in detritus and protected from predators. When these whelks mature they migrate inshore where suitable substrates for egg laying are available, such as rocks and macroalgae (Lebour, 1937).

A survey of the distribution of *Nassarius reticulatus* around the Isle of Anglesey revealed well established populations along the west coast and less developed populations along the north coast. No whelks were present along the east coast of Anglesey, along the south-east facing Menai Strait and around the north-west areas of Holyhead harbour and the Inland Sea. The western Anglesey coastline is affected by prevailing SW winds, which transfer large amounts of carrion onto these shores, and at the same time, due to the increased water circulation and sediment re-suspension, the sediments in these areas are well oxygenated and contain less silt and organic material. It was observed that *N. reticulatus* prefers well or moderately sorted fine sand sediments, with a medium percentage of organic (<0.5%) and silt (<2%) content. The paucity of predators that was recorded along the west coast of Anglesey may also be an important factor for successful juvenile *Nassarius* survival. In addition, the presence of adjacent rocky outcrops, suitable for egg laying, is vital for the successful colonisation of an area. *N. reticulatus* is very well adapted to varying degrees of wave exposure, since well developed populations were recorded in a range of wave-exposed to sheltered areas. However, strong tidal currents and extremely wave-exposed rocky coastlines may constrain larval dispersion and inhibit recruitment of juveniles.

During the present study a significant degree of allometry (i.e. changes in shape) regarding shell size variables of whelks was observed, as commonly occurs in many species of gastropods in which shell growth ceases at maturity (Vermeij, 1980). In addition, variations in the morphology of *Nassarius reticulatus* inhabiting environments of different wave exposure around Anglesey were observed. Whelks from sheltered environments (i.e. Rhosneigr intertidal lagoon), where predation pressure is increased, had thicker and larger shells. Similar observations have been reported for many gastropod species such as *Bembicium vittatum* (Parsons, 1997a), *Nucella lapillus* (Crothers, 1973) and *Littorina sp.* (Johannesson, 2003). Larger shells are stronger than ones of smaller length, since the relative shell strength increases roughly with the 1/3 power of shell length (Tokeshi *et al.*, 2000), and thicker more robust shells are harder to break (Hughes & Elner, 1979). The shape of *N. reticulatus* was similar between sheltered, semi-wave-exposed and wave-exposed environments, which is a common feature in gastropods with long pelagic larval phases. Species with pelagic larvae are able to recruit into various habitats, and as a consequence strong local adaptation and formation of distinct ecotypes in contrasting habitats is prevented (Johannesson, 2003).

The recruitment, growth and mortality of *Nassarius reticulatus* in the intertidal lagoon and on the subtidal beach in Rhosneigr (SW Anglesey) were described by analyses of multimodal length-frequency histograms. The von Bertalanffy parameters were estimated after decomposing the population distribution into cohorts using either a graphical method (MPA) based on Bhattacharya (1967) or a computerised method (i.e. LFDA) based on a maximum likelihood statistical analysis. The VBG parameters estimated by the MPA analysis were in close agreement with growth data derived from a mark-recapture experiment. Computer programs, such as the LFDA analysis, are usually designed for fish populations in which the movement of individuals will average the impact of environmental variations (Grant *et al.*, 1987). Such methods may therefore be less appropriate for non-mobile invertebrate populations where large variations of recruitment can occur from year to year and/or where growth rates may fluctuate with time due to the effect of population density (Grant *et al.*, 1987).

The MPA analysis of the length-frequency distributions indicated that the larvae of *Nassarius reticulatus* hatch and settle during the summer months, and are recruited into the intertidal lagoon and the subtidal beach populations in Rhosneigr at a size of 7-8.9 mm between February and April the following year. Seasonal variations in the growth rate were indicated by the MPA analysis and were also observed in whelks that were maintained under laboratory conditions. The growth rate of whelks was highest during the summer months, when seawater temperatures were maximal, lower during autumn, and zero during the winter months. Previous studies have also indicated that the growth rate of *Nassarius* species is higher when the temperature is increased (*N. obsoletus*: Scheltema, 1964; *N. reticulatus*: Lambeck, 1984; Barroso *et al.*, 2005a; *N. festivus*: Morton & Chan, 2004). Decreases in growth rate have been related to the cessation of feeding activity during winter, due to the lower seawater temperatures and to the decreased availability of food (Scheltema, 1964). The natural mortality of *N. reticulatus* was higher during the summer months, probably due to the increased seawater temperatures and the greater reproductive output. A higher annual rate of natural mortality was estimated in the intertidal lagoon population, where higher seawater temperatures are encountered during the summer and lower temperatures occur during the winter, compared with the subtidal beach population. In addition, predation by crabs was greater in the sheltered intertidal lagoon.

The MPA length-frequency analysis of the population distributions indicated that whelks from the intertidal lagoon had a lower growth constant but reached a higher maximum asymptotic length than those from the subtidal beach. Differences in the growth patterns between *Nassarius reticulatus* collected from the intertidal lagoon and from the subtidal beach were also observed in the laboratory, although all the whelks were maintained under the same conditions for a period of 6-7 months. The growth rate of small whelks (<21 mm) in the laboratory was lower for intertidal lagoon animals, whereas the growth rate of larger whelks (>21 mm) was higher for the lagoon animals. The length-frequency analysis also indicated that the larger whelks grew faster in the intertidal lagoon. *Nassarius* from the intertidal lagoon attained a larger size (~26 mm) in the laboratory than whelks from the subtidal beach (~24 mm), as was also indicated from the MPA analysis. The von Bertalanffy growth curves fitted using the MPA analysis indicated a strong seasonality of growth for both the intertidal lagoon and subtidal beach populations. Growth in the intertidal lagoon decreased after the end of September and resumed at the beginning of April, whereas growth in the subtidal beach ceased more than a month earlier, in mid August, and resumed also a month earlier, after mid-February. Similarly, in the laboratory whelks that originated from the subtidal beach stopped growing about a month earlier than the ones originating from the intertidal lagoon. The differences observed in the growth patterns of natural *N. reticulatus* populations from the intertidal lagoon and the subtidal beach are maintained even when the animals were reared under similar controlled laboratory conditions.

Morton & Chan (2004), also observed that the growth rate of *N. festivus* was significantly different between 3 different habitats, and indicated that the same differences in growth rate were maintained when whelks from the three sites were held under the same laboratory conditions.

Besides the seasonal and regional variations in the growth rate of *Nassarius reticulatus* described above, the growth rate of *N. reticulatus* may also be dependant on other factors, such as maturity stage, gender and food availability. Growth rate of *N. reticulatus* is size dependent: smaller whelks (<15, and between 15-20 mm) grew faster and for a longer period than large whelks (20-25 mm), while growth ceased in whelks from the biggest size classes (>25 mm). Shell growth of *Nassarius* species decreases abruptly after the whelks become sexually mature, probably due to a strategic shift of energy allocation from growth to reproduction (Scheltema, 1964; Tallmark, 1980; Morton & Chan, 2004). Smaller individuals that invest a large amount of energy into shell growth grew faster in laboratory conditions where the available food supply was presumably higher, compared with marked and recaptured individuals that were released in the intertidal lagoon. In sexually mature whelks, where most of the assimilated energy is invested in reproductive activity rather than shell growth, the growth rate under conditions of excess food supply (laboratory conditions) was similar to the growth rate under restricted food supply (field conditions). No significant difference was found in the present study between the growth of male and female *N. reticulatus* though females attain a significantly greater length than males. Similar growth rates between the two genders have also been reported for other gastropod species such as *Ilyanassa obsoleta* (Curtis, 1995), *Polinices duplicatus* (Edwards & Huebner, 1977) and *Concholepas concholepas* (Rabi & Maravi, 1991). Limitations in food availability suppressed shell growth of *N. reticulatus* though deposition of shell material on the shell edge of starved whelks was still in progress, since the shell lips were thinner during summer and thicker during winter.

Utilisation of the growth rings in the statoliths of gastropods is a recently developed technique used to estimate the age and growth of individuals. A few previous studies have determined a relationship between shell length and statolith diameter in order to back-calculate the shell size of an individual at the formation of a particular statolith growth ring. However, lack of experimental data in previous studies has not allowed validation of this method and the establishment of the periodicity of the statolith growth rings. In the present study the periodicity of formation of growth rings in the statoliths of *Nassarius reticulatus* was determined using larvae and juveniles of known age reared in the laboratory. *N. reticulatus* has two elliptical statoliths each one located inside a separate statocyst. Microscopical examination of the statoliths revealed a clear pattern of 1 to 7 concentric rings around the nucleus, which has a mean diameter of 5 um and is formed during the early embryonic stages. The statolith diameter found in developing embryos of *N. reticulatus* enclosed in egg capsules was 12.5 um, and by the time the larvae had hatched the diameter had reached 17.5 um. The formation of the first ring in the statoliths of *N. reticulatus* occurs during the stage of larval metamorphosis and settlement, possibly as a result of the shift from a pelagic to a benthic habitat. The mean diameter of the first prominent ring observed in the adult statoliths (33.2 um) was similar to the statolith diameters observed in the larvae of known age one day after settlement (34 um). A clear ring was observed in statoliths of larvae of known age only after they had metamorphosed and settled. The formation of the first ring is possibly correlated with the hatching phase in gastropods that are characterised by direct development (e.g. *Buccinum undatum*, Karageorgopoulos, 1997; *Nucella lapillus*, Saurel, 2002), whereas in species that spend a period of time in the plankton before they metamorphose (e.g. *Polinices pulchellus*, Richardson *et al.*, 2005a; *N. reticulatus*, Barroso *et al.*, 2005b; present study) the formation of the first ring is correlated with the settlement phase.

The prominent rings in the statoliths of *Nassarius reticulatus* following the settlement ring are formed annually, as was demonstrated in the statoliths from whelks of known age. The statolith diameter of 270-day old juveniles (70 um) was significantly smaller than the mean diameter of the first ring in adults (82.7 um), whereas the statolith diameter of 500-day old juveniles (93.6 um) was similar to the first ring diameter in adults. Thus it was concluded that the first ring following the settlement ring was formed after the first year of life. In the present study it was observed that formation of the dark ring in the statoliths of *N. reticulatus* occurred between late summer and winter, when statolith growth slowed down, probably due to reproduction initially and in response to cooler seawater temperatures later. Subsequently, formation of the light wider ring occurred between spring and early summer when most of the assimilated energy was allocated to somatic growth. A series of weaker lines have been observed in the statoliths of predatory gastropods, such as *Nucella lapillus* (Saurel, 2002), *Neptunea antiqua* (Richardson et al., 2005b) and *Polinices pulchellus* (Richardson et al., 2005a), but not in the statoliths of the scavenger *N. reticulatus* (Barroso *et al.*, 2005b; present study). Since there was no evidence for a daily periodicity of these weaker rings, Richardson *et al.* (2005a) suggested that these lines could be correlated with the feeding lifestyle of predatory species. The energetic cost of drilling prey shells, sometimes up to several days, may divert energy away from statolith deposition, thus producing weaker rings (Richardson *et al.*, 2005a).

An exponential relationship, which included measurements of larvae of all ages, juveniles and adults within a range from 0.3 to 30 mm was established between shell length and statolith diameter of *Nassarius reticulatus* in order to reconstruct the life history and growth patterns of individual whelks. The generated equation was used to estimate the shell length of *N. reticulatus* at the formation of the statolith growth rings and these length-at-age data were used to construct von Bertalanffy growth curves. The same equation can be used for male, female or imposex affected *N. reticulatus*, collected during different seasons and from different locations around Anglesey. The growth patterns of male and female *N. reticulatus*, collected from different locations around Anglesey, were described. No significant differences were observed between the growth rates of female and male *N. reticulatus*, as has been indicated above during the monitoring of growth in the laboratory. Also no significant difference was observed in the growth rate between females and imposex affected females. The shell length of *N. reticulatus* larvae at settlement was similar between whelks collected from different areas around Anglesey, indicating that the larvae spend a similar period of time in the plankton before settling onto the sediment. However, shell length during the formation of the annual rings was significantly different between whelks from different locations, indicating that different environmental factors affect growth in different habitats.

The von Bertalanffy growth curves constructed from statolith length-at-age data indicated that adults collected from the sheltered Rhosneigr lagoon had the highest maximum asymptotic length and the lowest growth constant compared with other areas, whereas whelks collected from the wave-exposed Church Bay site had the lowest maximum asymptotic length and the highest growth constant. The laboratory growth experiments and the length-frequency analysis (see above) also indicated that *Nassarius reticulatus* collected from the intertidal lagoon had a slow growth rate, but attained a final large size. The formation of larger shells in sheltered environments offers effective protection against predation (Crothers, 1973; Parsons, 1997a). *N. reticulatus* from the sheltered Rhosneigr lagoon invested more energy into thickening their shells for protection against predation, as was confirmed by the morphological comparisons in chapter 2. Since there is a maximum rate at which calcification can occur, when more material is devoted to shell thickening less is available for linear shell growth (Trussell & Smith, 2000).

Since the shell growth of *Nassarius reticulatus* is increased at higher temperatures, new shell material is added on the edge of the shell during the summer, resulting in thinner shell lips. During winter, shell growth is reduced and the edge of the shell lip thickens. The same seasonality in thickening of the shell lips of *N. reticulatus* has been observed by Tallmark (1980) and Barroso *et al.* (2005a). In mature individuals, when spiral growth ceases, further deposition of calcareous material leads to the formation of a permanently thicker lip with a ridge of 6-9 longitudinally elongated teeth projecting into the opercular aperture (Fretter & Graham, 1984; 1994; Barroso *et al.*, 2005a). The thickness of the shell lips and formation of teeth-like sculptures on the inner lip have been directly linked to maturity in gastropods and have been extensively used as age indicators for *Strombus gigas* (Appeldoorn, 1988), *S. luhuanus* (Wada *et al.*, 1983), and *Nucella lapillus* (Hughes, 1986). In the present study *N. reticulatus* with thin shell lips had smaller statoliths and smaller shell lengths, whereas animals with thick shell lips and well-formed teeth had bigger statoliths and larger shells. The thickening of shell lips and the formation of teeth are morphological characters correlated with maturity; however, due to the fact that mixed age classes were present in each of the shell lip condition categories, shell thickness and formation of teeth can only be used as rough indicators of maturity. Shell length and shell lip thickness can be severely affected as a result of damage due to predation or erosion, while statoliths are completely protected and isolated from the external environment, thus information concerning the past history of the organisms cannot be modified by external damage.

During the present study it was confirmed that similar sized whelks had statoliths of similar size, independently of their growth rates that may be affected by variable environmental conditions. Shell growth of *Nassarius reticulatus* is well correlated with statolith growth, thus the description of the growth patterns and life history events can be revealed from statolith increments. Furthermore, estimation of shell length at a particular age from the statolith diameter at the respective annual rings, was validated using marked and recaptured animals. The shell growth of marked individuals, which was monitored throughout a period of two years after they were released into the natural environment, was not significantly different from the shell growth that was estimated from their statolith annual rings. The annual rings formed in the statoliths of *N. reticulatus* therefore provide an accurate method for estimating the age of the gastropods and to back-calculate length-at-age data throughout their lifetime.

The MPA length-frequency analysis for the intertidal lagoon population identified only 5 age classes with the oldest individuals reaching a size of 27-28.9 mm, whereas the estimation of shell length based on statolith rings revealed 7 age classes with the oldest individuals attaining a length of 24.5 mm. Since the estimation of length-at-age data using statolith rings was well correlated with the growth increments measured in marked and recaptured whelks, it is suggested that the use of statoliths for the determination of age and growth of *Nassarius reticulatus* is a more reliable method than the analysis of length frequency distributions which resulted in overestimated growth rates. Similarly, several studies that have used analyses of size-frequency distributions indicated the unsuitability of those methods for the study of gastropod growth throughout the size range of the animal (Moore, 1937; 1938; Blackmore, 1969; Feare, 1970b). The overlapping of cohorts in older animals where growth rates are decreased (Blackmore, 1969), the erosion of the apical end of the shell that may mask small increases in shell length (Ekaratne & Crisp, 1984), size-dependant mortality and predation (Ekaratne & Crisp, 1984) and the seasonal or spatial variations in recruitment of a population can lead to erroneous estimates of growth rate when length-frequency analysis is used. Since length frequency distribution analyses can only detect gross changes in growth rates (Ekaratne & Crisp, 1984), such methods are only suitable for short-lived rapidly growing species where little or no overlapping of annual cohorts occurs.

In this study the spawning behaviour and the reproductive output of *Nassarius reticulatus* have been described. The egg capsules of *N. reticulatus* in the Rhosneigr intertidal lagoon were found attached on the underside of the stipes of red algae that were overhanging the sides of the rocks, thus promoting coverage of the egg capsules by the algal fronds and protection against desiccation and predation. Egg caspules were mainly laid on *Chondrus crispus*, occasionally on *Furcelaria lumbricalis* and *Plumaria plumosa*, but were never found attached to bare rock surfaces. *C. crispus* was highly abundant in the intertidal lagoon and its leathery, narrow and unbranched stipe offered the most ideal substratum for egg capsule attachment. Barnett *et al.* (1980) suggested that brown algae are a less favourable substrate for egg deposition than red algae, since they are covered by a thicker layer of mucilage which might constrain egg capsule deposition. In the laboratory tanks, where no algae were present, *N. reticulatus* successfully laid egg capsules on the tank surfaces, mostly under the slots of the tank lid through which the seawater circulated.

Breeding size classes of *Nassarius reticulatus* have a shell length of 21 mm or greater. Whelks smaller than 20.9 mm did not produce any egg capsules in the laboratory but grew faster than the larger size classes. Larger whelks (25-28.9 mm) produced more and bigger egg capsules·female^{-1}·day^{-1}, with more and bigger eggs·capsule^{-1}, and spawning occurred for a longer period of time, although less energy was devoted to growth. A shift in the energy allocation from growth to reproduction after sexual maturity has also been observed in many other gastropods, such as the estuarine nudibranch *Tenellia adspersa* (Chester, 1996), the saccoglossan opisthobranch *Olea hansineensis*, (Chia & Skeel, 1973) and *Littorina rudis* (Roberts & Hughes, 1980). Limitations in food supply clearly had a negative effect on fecundity and duration of spawning by *N. reticulatus* in this study. Fewer and smaller egg capsules with fewer and smaller eggs were produced when whelks were maintained under a limited food supply. When the whelks are under conditions of reduced food availability more energy is invested in metabolic maintenance and less in reproduction, so that these whelks will increase their chances of survival until the next breeding period (Cheung & Lam, 1999). This strategy may be an adaptive mechanism for scavengers which often face ephemeral and spatially unpredictable food availability.

Deposition of egg capsules by regularly fed whelks was recorded in all months of the year under laboratory conditions, even at seawater temperatures as low as 7°C. Reproductive activity increased in spring and summer, with peak fecundity during June, and a gradual decrease in early autumn. Although very few egg capsules were produced between September and October, spawning activity resumed after November and continued throughout the winter. Breeding of *Nassarius reticulatus* throughout Europe mainly takes place during spring and summer (March-August) (Rasmussen, 1973; Barnett *et al.*, 1980; Fretter & Graham, 1984; Barroso & Moreira, 1998). However, Lebour (1931) reported that egg capsules were produced throughout the year except

October and November in Plymouth. Long spawning seasons may be the result of different age groups or individuals spawning at different times (Wigham, 1975).

The egg capsules of *Nassarius reticulatus* produced during the summer and early autumn at warmer temperatures were smaller and contained smaller eggs than those produced during the early spring and winter, although the number of eggs per capsule was constant between different seasons. It is suggested that due to the increased level of egg capsule production in summer less energy is available for the construction of each individual egg capsule, thus smaller capsules and smaller eggs are produced in order to maintain a constant number of eggs·capsule^{-1}. Assuming that organisms have a finite and limited amount of energy available for reproduction, then they will only be able to increase the production of eggs if they produce smaller eggs (see reviews by Vance, 1973; Smith & Fretwell, 1974; Stearns, 1976; Bridges, 1993). The duration of the *N. reticulatus* embryonic development inside the egg capsules is longer during winter and spring when the seawater temperature is lower. The larger and heavier egg capsules produced during winter are usually stronger and more resistant to predation risks that occur during their prolonged embryonic development (Hughes, 1986). A reduction in the amount of capsular material (i.e. smaller and lighter weight capsules) during the summer may promote faster erosion of the capsule, thus allowing larvae to hatch at a smaller size when the temperature conditions are favourable (Chester, 1996). During the present study smaller eggs, which were produced at higher temperatures during summer and early autumn, had a shorter embryonic development and hatched into smaller larvae, compared with those hatched from larger eggs produced during the early spring and winter.

Hatching of *Nassarius reticulatus* larvae from capsules laid in the laboratory occurred successfully over a range of seawater temperatures (8.5-20°C), which occur in the natural environment during the spawning period of *N. reticulatus*. The duration of embryonic development increased as the seawater temperature declined. The shortest embryonic development period occurred at seawater temperatures of 17.5 and 15°C, which were within the temperature range recorded in the laboratory tanks and in the intertidal lagoon during the peak reproductive period in June. Similar variation of embryonic development duration with seawater temperature has been reported for *N. trivittatus* (Pechenik, 1978b) and *N. reticulatus* (Tallmark, 1980).

The recently hatched larvae of *Nassarius reticulatus* are characterised by a smooth transparent shell consisting of one whorl and a ciliated bilobed velum with a continuous brownish border along the inside of the margin. The swimming pattern of larvae was characterised by a passive sinking phase in the water column, followed by an active upward swimming phase towards the water surface, which became smoother as the larvae grew older. Recently hatched *N. reticulatus* larvae were photo-indirect and geo-negative. A negative geotactic swimming behaviour during the early stages would ensure that the larvae are concentrated in regions where food is abundant, and also the more rapid surface water currents would provide most favourable conditions for dispersal (Cragg, 1980). During the second week after hatching the development of larval eyes was complete, and larvae were strongly photo-positive, especially before they were fed. The young photo-positive larvae spent most of their time near the water surface where food availability was higher. The younger larvae of the intertidal/shallow sublittoral *N. reticulatus* were immediately aggregated close to the water surface after a 0.2 bar pressure increase. Intertidal and shallow sublittoral species are much more sensitive to pressure reductions because they face an increased danger of sinking and becoming trapped in the sediment, while larvae found in deeper water are able to range over a considerable vertical distance and are less sensitive to pressure (Cragg, 1980). As the *N. reticulatus* larvae were approaching metamorphosis they exhibited a geopositive swimming behaviour and spent more time closer to the bottom of the experimental beaker exploring the sediment. Older larvae were less sensitive to pressure increments. When the larvae were ~1 month old the velum became four-lobed and the foot was well developed. Just before metamorphosis (3-4 days) the anterior lobe of the foot expanded into two horns and the veligers were able to swim or crawl at will (swimming/crawling stage). Metamorphosis of *N. reticulatus* was complete when the velum was lost and at the same time the foot and radula were completely developed, at which time the juvenile whelks were fully adapted to a benthic lifestyle.

Since adult *Nassarius reticulatus* have a prolonged breeding period throughout the year and larvae are released from egg capsules under a wide range of temperatures, veligers will also experience a wide range of temperatures during their development from hatching to metamorphosis. Larvae that hatched during the colder winter months died long before achieving a sufficient size for successful settlement and metamorphosis, whereas larvae hatched during early spring reached the crawling/swimming stage but again did not metamorphose successfully. Larvae that were released during the summer and early autumn successfully settled and metamorphosed at a size close to or >700 um, 28 to 51 days after hatching depending on season. Optimum larval development occurred at temperatures 16.3-17.5°C, where larvae reached a larger size before metamorphosis and over a shorter period. Larvae developed at temperatures >17.5°C metamorphosed successfully but their growth rate from hatching to metamorphosis was lower. Even though the larvae released during winter and early spring were bigger at hatching, which might have given them an advantage for better development at low temperatures, they still did not settle. The smaller summer larvae had a faster growth rate and were more competent for metamorphosis than larvae which were larger at hatching. Scheltema (1967) indicated that the temperature required for metamorphosis can be higher than the efficient temperature for growth. Therefore, larvae hatched at low temperatures during spring were competent for metamorphosis but never achieved settlement, because the temperature was insufficient to promote all the morphological changes that occur during metamorphosis. Larvae hatched in summer and early autumn grew as the temperature was increased and synchronised their metamorphosis with the higher temperatures occurring during that period.

When larger winter larvae were developed under an artificial optimum temperature of 17.5°C they did metamorphose successfully; however, they delayed their metamorphosis for up to 40 days and settled at a much larger size (~1000 um), than the small summer larvae. These larger winter larvae were produced at a low temperature (12.4°C), and even though they were gradually acclimatised at 17.5°C, the temperature variations they encountered may have caused their delayed metamorphosis. A similar delay in metamorphosis was observed in the small summer larvae that were developed without the addition of sediment in larval culture. Although *Nassarius reticulatus* larvae metamorphosed even in the absence of sediment, the lack of a suitable substrate for settlement reduced their growth rate and delayed their metamorphosis. The ability to delay metamorphosis and extend planktonic life should increase the potential for dispersal, and therefore increase the probability of encountering a suitable habitat (Pechenik, 1980).

The mortality of *Nassarius reticulatus* larvae was very high during the first developmental stages of planktonic life. Survival of larvae was higher at temperatures of 20°C for the first 15 days after hatching, but more larvae survived until the final stages of metamorphosis at 17.5°C. Only 8% of the veligers survived from hatching to metamorphosis at 17.5°C, and half of them (4%) survived for a further 14 days after settlement. The heaviest mortality that most invertebrate species experience occurs during their early developmental period (Vance, 1973). Thorson (1950) indicated that important causes of mortality during the pelagic veliger phase of marine benthic invertebrates are lack of food, adverse temperature conditions, failure to find a suitable substrate, transport by currents and predation. Despite the extremely high larval mortality, the production of large numbers of larvae are sufficient to ensure population sustainability (Jablonski & Lutz, 1980). The larvae of *N. reticulatus* hatched under ambient summer temperature in the present study were successfully raised in the laboratory up to the age of 1.4 years (500 days). The growth rate of juveniles under ambient temperature was ~22% lower than the growth rate of larvae during their planktonic period. Juveniles were able to scavenge on fish or mussel flesh when they were about 6 months old.

A predominance of females is quite common in gonochoristic molluscs (Fretter & Graham, 1994). The percentage of female *Nassarius reticulatus* around Anglesey ranged from 60% to 91.2%. High imposex levels may be responsible for a reduction of females in a population due to increased mortalities (Feare, 1970a), as has been observed in *Nucella lapillus* and *Ilyanassa obsoleta* (Bryan *et al.*, 1986). However, no significant differences in the sex ratio between high and low imposex affected populations around Anglesey were found during the present study.

The percentage of imposex affected female *Nassarius reticulatus* around Anglesey ranged from 8.3% to 83.3% and the RPL index ranged from 9.2% to 48.2%. The highest incidence (83.3%) was observed in *N. reticulatus* from the north facing Cemaes Bay where boating activity is moderate to high and a small port with several fishing and leisure boats is present. A relatively high imposex incidence (42.4%) and the highest RPL (48.2%) were observed in whelks from the nearby area of Carmel Head-Cemlyn Bay, which is characterised by moderate boating activity. The percentage of imposex affected females was also high at Church Bay (40%), which is located on the north-west coast of Anglesey, in close proximity to the port of Holyhead. The north-east direction of the residual currents along the coast of Anglesey may transfer TBT-polluted water masses from Holyhead to the north-western and northern coastal areas around Anglesey. The percentage of females affected with imposex was lower than 21.3% for all the sites located along the south-west coast of Anglesey, where no ports or marinas were present and where the boating activity is moderate to low. The lowest RPL (9.2%) was recorded in Malltraeth, where boating activity was low and only 18.6% of females were affected by imposex. The mean VDS index ranged from 2.4 to 3.6 and there was no significant difference between the Anglesey populations, with the exception of Rhoscolyn (only 1 imposex affected female at VDS =1). Only a few females (3.03%) from the area Carmel Head to Cemlyn Bay were classified at the 1b VDS stage and no gastropods in 3b stage were found. No imposex affected females of advanced VDS stage 4+, and no sterile females with signs of vulva blockage carrying aborted egg capsules were reported during the present study. A clearly convoluted oviduct (AOS=2) was observed only in one highly imposex affected female (VDS=4) from the area around Carmel Head and Cemlyn Bay, which was characterised by a high imposex incidence (42.4%) and also by the highest RPL (48.2%). The *N. reticulatus* populations around Anglesey in 2004 appear to be less affected by TBT pollution, compared with populations in France (Stroben *et al.*, 1992b), Portugal (Barroso *et al.*, 2002), Spain (Barreiro *et al.*, 2001) and SW England (Bryan *et al.*, 1993) which had been examined in previous years and were characterised by higher imposex incidence, RPL, VDS, and AOS, as well as by the presence of sterile females carrying aborted egg capsules.

The imposex indices of sympatric populations of *Nucella lapillus* and *Nassarius reticulatus* were examined in order to compare sensitivity of the two indicator species. The imposex incidence of *N. lapillus* displayed a similar pattern to *N. reticulatus* with higher I%, RPS and VDS found in Cemaes Bay, Church Bay and Cemlyn Bay, whilst dogwhelk populations along the west Anglesey coast the RPS were less affected by TBT pollution. However, in most of the sites where imposex was examined for both species *N. lapillus* exhibited a higher imposex incidence. *N. lapillus* has been documented as a species very sensitive to TBT pollution by several investigators (Stroben *et al.*, 1992a; 1992b; Bryan *et al.*, 1993). According to Barroso *et al.* (2000) the differences in imposex levels between *N. reticulatus* and *N. lapillus* are related to differences in habitat type and diet type between the two species. *N. reticulatus* accumulates only 36.9% of the *N. lapillus* TBT-Sn body burden under the same exposure conditions (Stroben *et al.*, 1992a) and degrades TBT more efficiently than *N. lapillus* (Bryan *et al.*, 1993). A major advantage of using *N. reticulatus* as an imposex indicator species is its wider geographical distribution compared with *N. lapillus* (Stroben *et al.*, 1992a). In addition, imposex affected populations of *N. lapillus* populations were declining or were already absent from heavily polluted areas in southern England, France and Norway by the early 1990's, whereas *N. reticulatus* is able to recolonise polluted areas due to the higher dispersal ability of its planktonic veliger (Stroben *et al.*, 1992a). Stroben *et al.* (1992a) proposed that both *N. reticulatus* and *N. lapillus* should be used as TBT bioindicators species when both are found sympatrically, thus resulting in a broader database on the effects of TBT pollution (Stroben *et al.*, 1992a).

Comparisons of the results of the present study with older studies indicated a significant recovery of the *Nucella lapillus* populations around Anglesey from TBT pollution during the last 14 years although organotin pollution still remains an issue of concern around the British coasts. Bryan *et al.* (1993) indicated that even after restrictions on TBT usage were applied in 1987, the percentage of imposex affected female *Nassarius reticulatus* in SW England declined very slowly, due to the longevity of the species, the very slow decline, if any, of the penis length in older females and the limited recruitment of less-affected females (Bryan *et al.*, 1993). Even after the decline of TBT in seawater, exposure of juveniles to polluted sediment or food, where TBT degradation is slower than in the seawater, enhanced the development of imposex (Bryan *et al.*, 1993). Since waters leaving highly contaminated areas may carry high levels of TBT for great distances, TBT pollution from commercial shipping may affect areas not immediately adjacent to major ports (Barreiro *et al.*, 2001). Regulations for further restrictions on the use of TBT antifouling paints were considered and a global prohibition on the application of organotin compounds agreed. After January 1, 2003 no ships could apply or re-apply organotin compounds which act as biocides in antifouling systems, and by January 1, 2008 ships have either to remove such compounds from their hulls or external parts or surfaces, or apply a coating that forms a barrier to such compounds leaching from any underling non-compliant anti-fouling systems (source: http://www.imo.org/home.asp). It now remains to be seen whether the levels of imposex observed in *N. reticulatus* and *N. lapillus* will continue to decline following the introduction of a full ban on the use of TBT in 2008.

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