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Settlement and bioadhesion of two marine fouling organisms, Pomatoceros lamarckii and Laminaria digitata

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Settlement and Bioadhesion of Two Marine Fouling Organisms, *Pomatoceros lamarckii* and *Laminaria digitata.*

A thesis
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by

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in candidature for the degree of
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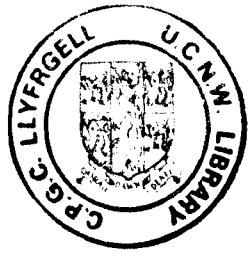

"All nature is perverse & will not do as I wish it"

Charles Darwin.

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Dare I say that embarking upon a Ph.D. degree is somewhat akin to serving an apprenticeship? As the way in which the development and progress of an apprentice depend a great deal upon the skill of the master craftsman to whom he is apprenticed so also does the Ph.D. student draw heavily upon the knowledge and skills of his supervisor. My most sincere thanks must, therefore, go to Dr. Graham Walker for his enthusiasm, help, advice, encouragement, stimulating discussion and the unstinting use of his time at all stages of the project. It would be difficult to envisage a more thorough or conscientious mentor.

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Summary

An investigation of certain aspects of the biology of *Pomatoceros lamarckii* and *Laminaria digitata* was carried out with particular reference to the factors influencing the settlement of *P. lamarckii* larvae and to the bioadhesion of both species.

The larvae of *P. lamarckii* were found to be negatively phototaxic throughout their development. A distinctive pattern of settlement behaviour was observed and as the larvae settled they became attached to the substratum by a mucus pad situated in the region of the ventral shield epithelium. Larvae settled gregariously on and close to conspecific adults, but the presence of conspecific juveniles did not induce settlement. Unsuccessful attempts were made, using whole and parts of animals and tubes in a range of solvents, to isolate a chemical settlement stimulus and it was concluded that it is highly unlikely that a chemical cue is produced by *P. lamarckii* adults. Biofilming was found to be the major factor in inducing settlement and it was demonstrated that substrata biofilmed in the presence of *P. lamarckii* adults attracted significantly more settlers than did substrata biofilmed in other ways. The adult mediated biofilms were found to contain large numbers of a rod-shaped bacterium which was present only in very low numbers in the other biofilms and it is proposed that this bacterium, or its exopolymers, may provide the primary settlement stimulus. Natural settlement was examined on pebbles at three different locations and the results compared with the laboratory findings.

Tenacity was measured for the tubes of *P. lamarckii* and the haptera of *L. digitata*. A mean tenacity of $24.75 \times 10^5 \pm 6.7$ N m^{-2} was recorded for *P. lamarckii* and a mean tenacity of $4.21 \times 10^5 \pm 1.9$ N m^{-2} was recorded for *L. digitata*.

Chapter 1

The Settlement and Bioadhesion of *Pomatoceros lamarckii* and *Laminaria digitata*

A Literature Review

GENERAL BIOLOGY

Pomatoceros species and *Laminaria* species are important fouling organisms which readily colonise man-made structures such as piers, docks, buoys, offshore oil rigs and ships hulls. A better understanding, both of the way in which the larvae and spores of these organisms attach to and settle on man-made structures and of the way in which adults of these species adhere to substrata is a prerequisite to the search for improved anti-fouling techniques.

Pomatoceros species (Polychaeta: Sabellida: Serpulidae) are sedentary polychaete worms which have a wide geographic distribution over the Atlantic and Pacific oceans and from low to high latitude. They have also been recorded from low water to the abyssal depths (Zibrowius, 1977). There are two Atlantic species; *Pomatoceros lamarckii* and *Pomatoceros triqueter*.[1] *P. lamarckii* is to be found mainly in the littoral zone and *P. triqueter* in the sub-littoral (Zibrowius, 1968). The calcareous tubes of adult *P. lamarckii* are a common sight on the rocky shores of the British Isles, where they are often to be found in profusion on rocks, stones and the shells of other marine invertebrates. *Pomatoceros* species are dioecious

[1] See chapter 2.

and fertile adults may be found in Britain at all times of the year, with the greatest numbers occurring between the months of May and August. Adults are initially male, later becoming female (Føyn & Gjøen, 1954). Gametes are released into the water column where fertilisation takes place and in *circa* 16 hours free-swimming, planktotrophic trochophore larvae develop. In the laboratory the larval phase can last at least eight weeks (Segrove, 1941). *Pomatoceros* larvae are able to tolerate a wide range of salinities from 18‰ to 34 ‰ (Moat, 1985) and this salinity tolerance, coupled with long larval existence, undoubtedly contributes to the wide distribution of the animal.

The general biology of *Pomatoceros triqueter* [1] was very thoroughly investigated by Thomas (1940), in a comprehensive study of *Pomatoceros, Sabella and Amphitrite* and Segrove (1941), who described the larval development of *P. triqueter*, covering development from the egg. This study included microscopical investigation of the structure of the larval stages, metamorphosis and settlement. Several stages of metamorphosis were described but these stages were disputed by Føyn & Gjøen (1954) who considered them to be abnormal. Segrove's work was largely repeated and confirmed by Moat (1985) as part of his doctoral study. Moat, however, found the larvae just prior to settlement to be photonegative, whereas Segrove maintained that they were photopositive. Moat also investigated the temperature and salinity tolerances of *P. triqueter* larvae. His results show tolerance to a wide range of temperatures and salinities with optimum conditions for development of 34‰ salinity and 20°C water temperature. These figures confirmed and more precisely defined Segrove's earlier findings.

[1] It should be assumed that all references to previous works on *Pomatoceros triqueter* are equally relevant to *P. lamarckii* as most earlier workers did not distinguish between the two species.

Laminaria digitata (Phaeophyceae: Laminariales: Laminariaceae.), the common kelp, is a North Atlantic species which extends from north of the Loire, south of Brittany, to the Kara sea and also round to Spitzbergen, Iceland, East Greenland, Newfoundland, Nova Scotia and as far as Cape Cod (Kain, 1979). This review also noted that around the British Isles *L. digitata* is to be found around the rocky shores in a belt extending from the extreme lower littoral to a depth of at least two metres below mean low water. This lower limit is quite variable throughout the range, ranging from two metres off the Isle of Man to a maximum recorded depth of twenty metres off Nova Scotia. This variation in maximum depth is thought to relate to competition with *L. hyperborea* which, in suitable conditions, will out-compete *L. digitata* (Kain, 1979). The mature sporophyte consists of a holdfast, a long, flexible stipe and a blade which tends to split into strips as it becomes older. The holdfast, which is made up of a number of root-like haptera, provides a very secure hold on rock, boulders, other plants and any firm, man-made structures. Blade growth is from a basal meristematic region which allows the plant to keep pace with the constant wear on the blade tip caused by tidal action and water turbulence.

L. digitata is oogamous and has a heteromorphic life history consisting of a macroscopic, diploid (sporophyte) stage (the adult plant) and a microscopic, haploid (gametophyte) stage (Bell & Woodcock, 1976.). These microscopic gametophytes are dioecious and have seldom been found in the field. Fertile sporophytes are to be found at all times of the year but are most abundant between June and October (Kain, 1979). Raised, discoloured patches (sori) appear on the blade of the fertile sporophyte. These sori contain the meiosporangia in which meiosis takes place to give haploid, bi-flagellate zoospores (Fig. 1). The zoospores settle and develop into dioecious, haploid gametophytes. Oogonia develop on the female

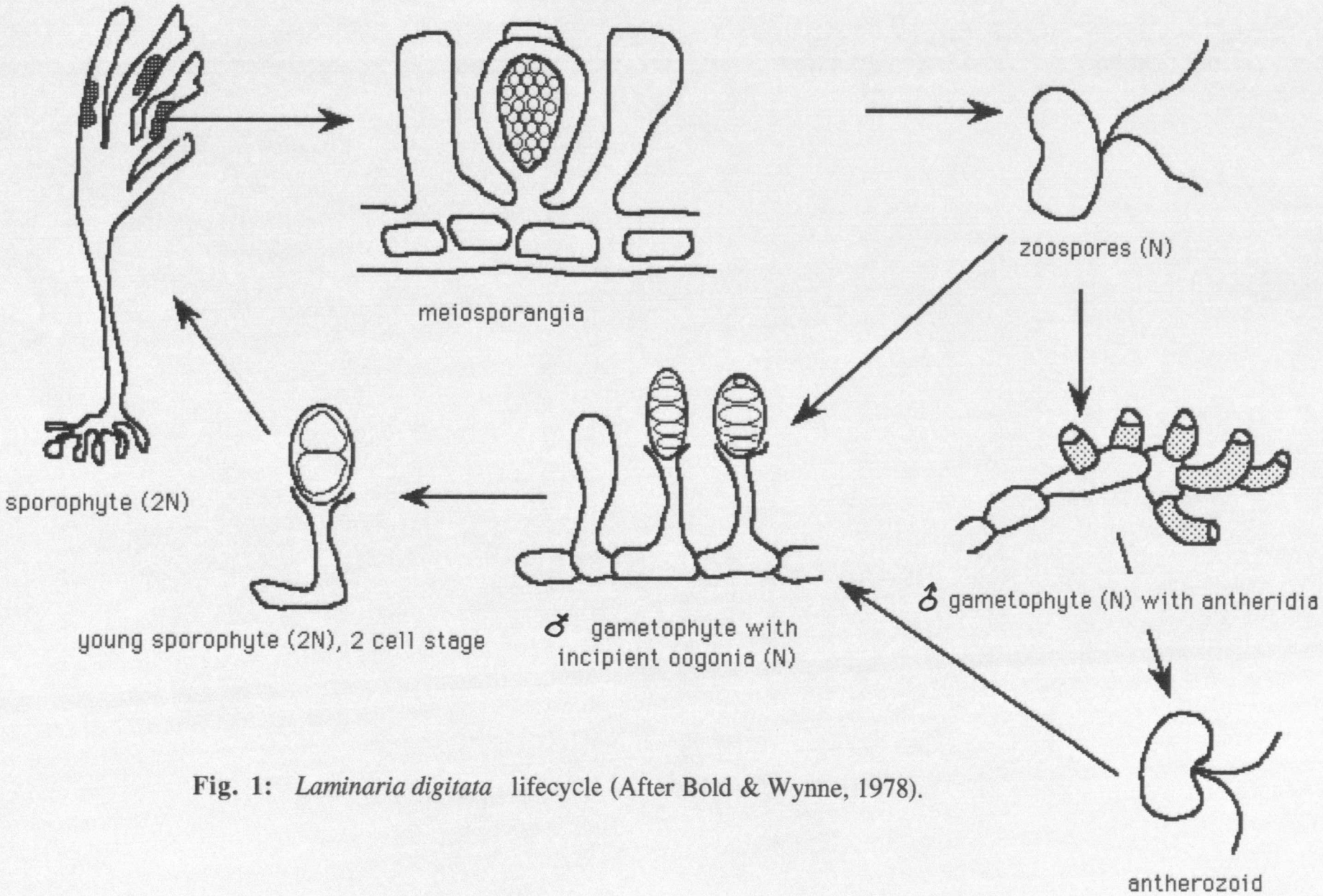


Fig. 1: *Laminaria digitata* lifecycle (After Bold & Wynne, 1978).

gametophytes, each containing a single oocyte. The male gametophytes develop terminal antheridia, each containing a single, bi-flagellate antherozoid. When fertilisation occurs, the diploid zygote, which develops into the sporophyte, remains attached to the female gametophyte for a little while during early cell division.

Reinke (1876) carried out an early investigation into hapteron growth, discovering that this growth was essentially apical. Yendo (1911) did further work on holdfast formation in very young sporophytes, describing the growth of 'rhizines' (rhizoids?) from an initial disc-like holdfast and the subsequent formation of primary and secondary haptera. Williams (1921) gave an account of the gametophyte stage of the life cycle and described the fertilisation of the oogonia.

Kain (1979), in a comprehensive review of the genus *Laminaria*, discussed structure, physiology, reproduction and laboratory culture. References to the biology and culture of *Laminaria digitata* included details of annual growth and periods of fertility, the morphology of the gametophyte stage and the morphology and settlement of meiospores. She also outlined the conditions necessary for the propagation of *L. digitata* from meiospores.

LABORATORY CULTURE OF *Pomatoceros lamarckii* AND *Laminaria digitata*

Pomatoceros species are not difficult animals to culture and ova and sperm can be obtained at any time of year, late spring to summer being the optimum period for obtaining the gametes. Such ease of culture makes *Pomatoceros* a very useful animal for research purposes. Segrove (1941),

Føyn & Gjøen (1954) and Moat (1985) all deal with the laboratory culture of *P. triqueter* larvae, their methods all being rather similar.

Laboratory culture of macroalgae has been well established for some time. Harris (1932) used laboratory culture in her experiments and Carter (1935) advocated the use of orange juice to promote the growth of filamentous gametophytes. It is interesting to note that Harris found sporophytes to be sexually mature from September until January which conflicts with the later findings of Kain (1979). Although culture methods follow a broad pattern there is considerable variation in the techniques of various workers. This variation centres mainly on the optimum irradiance and types of culture media used. Lüning & Neushul (1978), for example, grew filamentous *Laminaria* species gametophytes to sexual maturity at an irradiance of 6μE cm^{-2} sec^{-1} (approximately 3000 Lux), while Kain (1964) grew *Laminaria* species gametophytes to maturity at 33μg.cal cm^{-2} sec^{-1}. (approximately 20 lux) and Vadas (1972) used an irradiance of 100-200 foot-candles. (1076-2152 lux) to culture the gametophytes of *Nereocystis luetkeana.*

It can be seen that a direct comparison of irradiance levels used by different workers is beset with difficulty as workers tend to employ different units of measurement and these units are not always precisely interconvertible. One of the reasons for this difficulty of comparison is that units such as foot/candles and lux are units of illuminance but it is now generally agreed that, particularly in plant biology, the more suitable measurement is that of irradiance. This complex topic will be dealt with in chapter four but the following rough conversion guide may be used for purposes of comparison:

- 1W m^{-2} ≈ 5μE m^{-2} sec^{-1} ≈ 250 lux (Lüning, 1981).

Where W = Watt. μE = micro-Einstein.

Kain (1964) also investigated temperature requirements. Her results showed that meiospores were able to survive 40 days at 17°C and 60 days at 5°C and that low irradiance and unchanged medium resulted in filamentous gametophytes. Vadas (1972), in his work on *Nereocystis luetkeana* , judged light to be the single most important factor in sexuality and subsequent sporophyte growth. Temperatures between 10°C and 15°C were not found to be critical but at 20°C gametophytes did not mature. Bolton & Lüning (1982) found that *Laminaria digitata* grew well over a wide range of temperature in the laboratory, with optimal growth at 10°C. They also found that temperature tolerance is low. 21°C is the maximum temperature at which the sporophyte will survive, plant disintegration occurring at 23°C. Sundene (1962), working in Oslofjord, Norway, transferred young *L. digitata* sporophytes, *circa* 1-2 cm long, onto concrete blocks, tied them down with nylon fishing line, and grew them on in the sea. The experiment was commenced in the autumn. Results showed that there was no perceptible growth in the summer and that annual growth started September-October, was slow during the autumn, increased in winter and was rapid in spring when maximal length was attained.

SETTLEMENT AND ADHESION OF *Pomatoceros lamarckii*

There is no information in the literature which deals in any meaningful way with the adhesion of *Pomatoceros lamarckii.* Faouzi (1931) made an early study of *P. triqueter* tube formation in which he noted that the tube was incomplete, the substratum forming the base. He also determined that the dorsal ridge, which is a characteristic feature of all *Pomatoceros* species tubes was produced by a prominent mid-ventral fold in the collar. Robertson & Pantin (1938) gave a short account of factors

affecting tube formation and observed that the animal was apparently able to produce calcium carbonate in artificial seawater in the absence of a dietary source of calcium.

Swan (1950) made the first in-depth study using radio-active strontium as a tracer. He found that calcium is partially taken up directly from seawater into the tube without intervention by the worm. He also found that at least part of the calcareous material is secreted to the outside of the animal by the major subcollar glands. Hedley (1956), in his introduction, implied that the direct take-up hypothesis is incorrect. He gave no reason for this implication but showed the existence of the exocrine tubulo-racemose glands responsible for the production and secretion of calcium carbonate. However, Neff (1971) reaffirmed the view that serpulids are able to obtain some of the calcium required for tube construction directly from seawater. Simkiss & Wilbur (1989) stated that when the decalcified acid mucopolysaccharide matrix of a *Pomatoceros* species tube was kept in a solution containing calcium carbonate and bicarbonate ions the matrix became re-mineralised. This finding also supports the direct take-up hypothesis. Hedley (1958), working on tube formation by *P. triqueter*, stated that "a series of ridges on the outer surface of the tube give a false and superficial appearance of a series of growth rings." Recent research suggests that *Pomatoceros* species do in fact produce such growth rings but that these rings do not appear to be connected with tidal or other rhythms (Richardson, 1992, pers. comm.). Hedley (1958) also determined that *Pomatoceros* species tubes can be complete in cross-section and that adult serpulids lie ventral surface uppermost.

As the animal grows and the tube becomes longer, the older, weaker part of the tube usually suffers damage or erosion. The resulting open posterior end offers easy access for predators such as errant polychaetes, which are often to be found inhabiting old, abandoned *Pomatoceros* tubes.

In order to prevent such attacks *Pomatoceros lamarckii* builds a calcareous end-plate in its tube. Hedley (1958) determined that the mucous glands of the ventral epithelium also provided the calcareous material for the construction of this end-plate.

Segrove (1941) gave a comprehensive account of the development of *P. triqueter* which remains the classical account of *Pomatoceros* species larval structure. However, settlement information was limited to a very brief description of where the larvae settled. No reference was made as to how they settled or what influenced the choice of settlement site. Føyn & Gjøen (1954) disagreed with some of Segrove's work on metamorphosis and added a little more substance to the settlement observations. They noted that the settling larvae often initially attached by the posterior end. After settlement they secreted a fine, semi-transparent tube open at both ends. It was also noted that larvae tended to settle in small groups and favoured the junction of the base and sidewall of the settlement vessel. The main questions, however, still remained to be answered: how does settlement actually occur?, what influences the choice of settlement site and is there a settlement factor involved? The present study will examine these questions in some detail.

Although little work has been done on *Pomatoceros* species larvae there is a considerable body of work available on the settlement of other marine invertebrate larvae. Some of this work relates to closely associated species of sedentary polychaetes and highlights settlement factors which many marine larvae hold in common. Knight-Jones (1951), working on the settlement of *Spirorbis borealis* made several important discoveries. He was able to show that *S. borealis* larvae settled on glass or stone only if these substrata had algal films, that the majority settled on surfaces which bore their own species and that settlement took place earlier on surfaces which held previously settled individuals than on bare control surfaces. He

observed that "gregariousness .....is probably a general feature of the settlement behaviour of planktonic larvae, helping them to find suitable habitats, to maintain old breeding stocks and to form new ones." Working on the barnacle *Elminius modestus* Knight-Jones and Stephenson (1950) showed that it too settled gregariously and Knight-Jones (1953), working on the barnacle *Balanus balanoides*, was able to add the facts that adult body fragments, when placed on various surfaces, made these surfaces more attractive for cypris settlement, but that actual contact with an adult shell was necessary for settlement to take place. However, it was left to Crisp & Meadows (1962) to demonstrate the presence of a chemical 'settling factor' which could be shown to influence the settlement of barnacle cyprids.

Wilson (1968) carried out pioneering work on the settlement of *Sabellaria alveolata*, a sedentary polychaete which forms its tube by cementing together particles of sand, in which he found that 'slimed', i.e. biofilmed, surfaces were not particularly conducive to settlement and that there was no preference for rough or smooth surfaces. He went on (Wilson, 1970) to show that tube fragments, crushed to sand, attracted larvae to settle preferentially and that physical factors have only a minor influence on settlement. Wilson was also able to show that the strongest stimulus for the larvae to settle was contact with the adult tube and that the chemical settlement factor which caused this stimulus was insoluble in water and unaffected by drying.

Nott (1973) produced a relevant paper on the settlement of *Spirorbis spirorbis* larvae. In addition to the detailed account of settlement and attachment he gave useful information on the preparation of such larvae for electron microscopy. Nott and Parkes (1975) described calcium secretion in the same species and mentioned in passing that aggregates of calcium have been observed in the gut of *Pomatoceros triqueter*.

In addition to Knight-Jones (1951), many workers have noted the effect of biofilming on larval settlement. e.g. Zobell & Allen (1935), Meadows & Williams (1963), Wilson (1968), Scheltema (1974), Maki & Mitchell (1985) and Maki *et al.* (1990). It is obvious that biofilming is an important factor in the settlement of the larvae of many, but not all, species of marine invertebrates.

Thorson (1964) collated data on the pelagic larvae of 141 invertebrate species. 82% of these larvae were found to respond positively to light in their early stages, becoming photonegative in later stages. One of the exceptions given was *Pomatoceros triqueter* which, he stated, was photopositive throughout the larval stage. This observation was, however, based upon the works of Segrove (1941) and Føyn & Gjøen (1954) both of which, it should be noted, gave only passing reference to light reactions.

In a major review of invertebrate larval settlement, Crisp (1974) brings together all the work to that date and provides a comprehensive overview of the subject. He provides many insights of his own on various topics and the work is also a valuable source of reference material. Finally, Scheltema *et al.* (1981), working on *Hydroides dianthus*, a species with many similarities to *P. triqueter*, found that larvae preferred to colonise surfaces inhabited by adults but that there was a wide variation in density of settlement.

ATTACHMENT AND ADHESION OF *Laminaria digitata*

So far as can be ascertained, very little work in this area has been done on *Laminaria digitata* and few papers which have any bearing on the topic have been uncovered. Delf (1932) carried out pulling tests on stipes of *L. digitata* but not on the holdfasts so her results have little to contribute to

the knowledge of the adhesion of the plant. Davies *et al.* (1973), in an ultrastructural study of the epidermal cells in the apical meristem region of the secondary haptera of *Laminaria* species, found that the dominant feature of the hapteron meristoderm cells is their active secretory role and that the meristoderm cells produce vast quantities of polyphenolic substances. Tovey & Moss (1978) made a scanning electron microscope study of the production of rhizoids by cut sections of *L. digitata* haptera and the way in which the rhizoids attach themselves to a variety of materials. The major conclusion was that the cells of the rhizoids grew to fill every microscopic variation in surface texture, building up an exact impression of the profile of the substratum. The rhizoids secreted abundant mucilage which acted as a bonding agent both to adjoining rhizoidal cells and to the substratum.

Stockton et al. (1980) carried out a mainly biochemical study of *L. digitata.* The results showed increasing amounts of polyguluronate alginates in blade, stipe and holdfast and it was suggested that this higher level of alginates was responsible for the increased rigidity of the haptera. Finally, Moss *et al.* (1981) looked at the colonisation by kelps on oil rig platforms. They suggested that such colonisation results from the drifting of young sporophytes. They found that the rhizoids appear to have no definite outer boundary to the cell walls and that mucilages appear to flow into the water and have the ability to 'set' after flowing into the irregularities of the substratum.

Although there is a lack of information on attachment and adhesion directly related to *L. digitata* work has been done on other species, some of which is relevant. Evans & Christie (1970) and Christie *et al.* (1970) found, at the apical dome of zoospores of *Enteromorpha* species, vesicles which contained either a component of an adhesive substance or a substance which triggers the release of an adhesive substance. They also found that

the settled zoospores secreted an adhesive of fibrillar appearance which was largely proteinaceous and was probably a mucoprotein or mucopolysaccharide. Chamberlain (1976) stated that algal spores would settle on both high and low energy surfaces. He went on to suggest that dissolved organic materials may be rapidly adsorbed to new surfaces to yield a low energy surface and that consequently, in the marine environment, all potential fouling surfaces may be reduced to a common low surface energy. Working on the spores of red, green and brown algae, Fletcher (1976) found that, in all the species examined, the basal rhizoidal (attachment) filaments were found to secrete a mucilaginous material from the apical regions which facilitated good contact with the substratum and functioned as an adhesive. Harlin & Lindbergh (1977) investigated the selection of substrata by macroalgae using textured plastic discs bolted to the rock on site and found that surfaces with greater relief were preferentially colonised.

Hardy & Moss (1979) looked at the effects of the substratum on the morphology of the rhizoids of *Fucus* species germlings and found that rhizoids were shorter and stouter on rough substrata and that a more secure attachment resulted from the greater surface area. They noted that mucus conforms to the surface shape and that rhizoids will penetrate weaknesses to increase their hold.

Norton (1983), working on *Sargassum muticum* , used a "water broom" at flow velocities of 57 cm sec^{-1} and 90 cm sec^{-1} to show that the tenacity of attachment increased with the length of time the germling had been "in residence" on the substratum. He was also able to show that germlings with rhizoids, no matter how small, adhered almost immediately. Just one attached rhizoid was sufficient to resist the strongest jet from a pipette. Older germlings showed an increased quantity of acid mucopolysaccharide around the rhizoids, making them much more difficult

to detach than zygotes even if both had been resident for the same length of time.

Fletcher & Baier (1984) found that critical surface tension affected the growth of rhizoids of *Enteromorpha intestinalis*. On substrata with a high critical surface tension the alga developed a compact, discoid rhizoidal base with short, much branched and tightly adjoined filaments, whereas on substrata with a low critical surface tension long, outwardly spreading free filaments developed. Adhesion was said to be stronger on the substrata with high critical surface tension but pulling tests to establish relative tenacities were not made.

THEORETICAL CONSIDERATIONS OF ADHESION

It is difficult to find any work encompassing the theory of adhesion which does not immediately launch into fairly high-powered mathematics and physics in order to explain the concepts. Fortunately, however, some texts are presented in a form more palatable to biologists e.g. Baier, 1970; Kinloch, 1980; Stork, 1980; Waite, 1992.

Of the several theories of adhesion which presently exist, the most widely accepted is the Adsorption Theory: "This theory proposes that, provided sufficiently intimate inter-molecular contact is achieved at the interface, the materials will adhere because of the surface forces acting between the atoms in the two surfaces. The most common such forces are van der Waals forces and are referred to as secondary bonds. In addition, chemisorption may well occur and thus ionic, covalent and metallic bonds may operate across the interface; these types of bonds are referred to as primary bonds" (Kinloch, 1980). In order to achieve the necessary inter-molecular contact an adhesive should have the ability to spread easily over

the bonding surfaces and to displace air or water and contaminants. If a surface has a low surface free energy, water dropped onto the surface will remain in droplet form and, if the surface is tilted, the water will easily run off, leaving a practically dry surface. 'Teflon' (polytetrafluoroethylene) non-stick surfaces are good examples of such surfaces. For adhesives to spread readily the substratum should have a high surface free energy. Surface free energy can be calculated from the contact angle of a water droplet with the surface in question (Fig. 2). When the contact angle becomes 0°

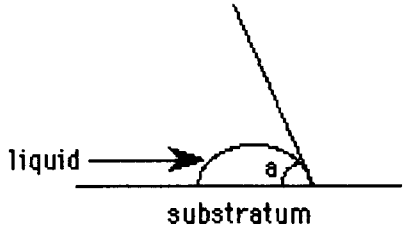


Fig. 2: The contact angle (a) of a drop of liquid at equilibrium. If angle 'a' > 0° the liquid is none-spreading. (After Kinloch, 1980).

the liquid spreads spontaneously (Kinloch, 1980) but Baier (1970) stated: "Water contact angle is notoriously unreliable as an indicator of relative surface energy, particularly.....for biological materials". Molecules in a fluid are held together by comparatively long range attractive forces. These forces are the forces of cohesion. The attractive forces between solid and fluid molecules (adhesive forces) are greater than cohesive forces. Consequently, when two solids bonded together by a liquid are pulled apart fracture is most likely to occur within the liquid. The force resisting this tendency to fracture is surface tension. The force achieved by surface tension is inversely related to the thickness of the fluid layer i.e. the thinner the adhesive, the stronger the bond. Impurities in a fluid, such as dust

particles, will result in a lower surface tension than that for the pure fluid, resulting in a weaker bond (Stork, 1980).

The formation of strong bonds underwater presents special difficulties which continue to pose problems in those areas where such bonds are necessary e.g. dentistry. Waite (1992) lists four adverse effects which water has on bonds:

- Water forms a weak boundary layer on the surface.
- Water invades the interface by crazing/cracking.
- Erosion of the adhesive is caused by hydrolysis.
- Swelling of the adhesive results from water absorption.

One of the most interesting and challenging aspects of marine adhesion is the way in which animals and plants easily achieve solutions to these problems, which still tend to defy the best efforts of man to mimic. It is hoped that the present study will add to the sum of knowledge of this subject.

Chapter 2

Pomatoceros triqueter or *P. lamarckii* ?

The Taxonomic Debate

Almost since the time that the Atlantic species of *Pomatoceros* was first described by Linnaeus (1767) as *Serpula triquetra* there has been controversy among taxonomists regarding the number of species which belong to the genus. Subsequent to Linnaeus, *S. triquetra* was recorded as *Pomatoceros tricuspis* by Philippi (1844), Leukart (1849) and Mörch (1863) before being redefined as three associated species; *Vermilia lamarckii, V. socialis* and *V. pusilla* by Quatrefages (1865) using body colour, branchial filament colour and segment number as the criteria for this division. Later workers ignored this division and chose to revert to a single species which was first described as *Pomatoceros triqueter* by Hansen (1878).

Zibrowius (1968) re-described the genus as two species, *Pomatoceros triqueter* and *Pomatoceros lamarckii,* based on the form of the operculum and bathymetric distribution. He described the distal end of the operculum of *P. triqueter* as 'cone shaped' and inserted laterally on the opercular stalk and that of *P. lamarckii* as 'cup shaped' and inserted medially on the opercular stalk. He makes no reference to the research carried out by Thomas (1940) who found that when the operculum was removed a new operculum, often with a differently shaped distal end, quickly regenerated. His field work, based on the criteria which he proposed, indicated that

Pomatoceros triqueter was to be found mainly sublittorally while *P. lamarckii* occurred predominantly in the littoral zone. This distribution conflicted with the widely held view that *P. triqueter* is the common species in the littoral zone. Moat (1985), working at Plymouth, England, reported that no representatives of *P. lamarckii* were to be found in the littoral zone. One further difference between the species has been detailed by Nelson-Smith *et al.* (1990) which is that the tube of *P. lamarckii* has an additional longitudinal ridge at the mid point on either side. However, workers in the field have continued to experience difficulty in distinguishing the two species with any degree of certainty and doubt still exists as to whether the genus *Pomatoceros* can in fact be separated into these two distinct species.

Ekaratne (1982) attempted to solve the problem by investigating the genetic make-up of those animals which satisfied the criteria laid down by Zibrowius (1968) in order to ascertain whether genetic differences between the species could be determined. Using horizontal starch gel electrophoresis techniques, she was able to show that genetic differences do exist between the species as defined by Zibrowius and also showed that, using these genetic differences as a guide to speciation, 97% of *Pomatoceros* found in the littoral region were *P. lamarckii* and 99% of those collected from the sub-littoral were *P. triqueter.* Ekaratne obtained viable larvae from experimental inter-specific crosses but discounted these experiments as the controls, which consisted of ova to which no sperm had been added, also yielded viable larvae in all experiments. This outcome suggested that there was experimental error and no further crossing experiments were attempted.

If Ekaratne had been able to show that interbreeding between the two species was not possible the case for two distinct species would have been very strong indeed. However, Tort (pers. comm.) has successfully cross-

fertilised the two species of *Pomatoceros* as defined by Zibrowius and has also successfully repeated the operculum regeneration experiments of Thomas (1940). Only slight differences in number and shape of the terminal spines were found and the general shape remains consistent with that of the original operculum. The present situation is, therefore, that there are two Atlantic species of *Pomatoceros* in which the morphological criteria for separation are somewhat ill-defined. These species can interbreed to give viable larvae but it is not known whether the F_1 generation is also fertile. The genetic variation shown by Ekaratne strongly supports the work of Zibrowius (1968) but is of little use to the field biologist.

In the course of this study the majority of animals found and used have conformed to the criteria described by Zibrowius (1968) for *P. lamarckii.* However, animals conforming to the criteria described for *P. triqueter* and other animals which showed a combination of characteristics, e.g. the extra ridge on the tube which is characteristic of *P. lamarckii* and the cone-shaped operculum of *P. triqueter,* were also found on various occasions. In view of the above observations and the fact that resolution of the taxonomic status of *Pomatoceros* is outside the remit of this study, no attempt has been made during the experimental work or within this thesis to distinguish any of the experimental animals of the genus *Pomatoceros* as separate species. However, as the available evidence strongly supports the case for *P. lamarckii* being the predominant species of *Pomatoceros* to be found in the littoral zone, all animals of the genus *Pomatoceros* which were used for experimental purposes will henceforth be referred to as *Pomatoceros lamarckii.*

Chapter 3

Some Observations on The Culture and Larval Stages of *Pomatoceros lamarckii*

INTRODUCTION

The calcareous tubes of adult *Pomatoceros lamarckii* (Polychaeta: Sabellida: Serpulidae) are a common sight on the rocky shores of the British Isles, where they are often to be found in profusion on rocks, stones and the shells of other marine invertebrates. *Pomatoceros* species are dioecious and fertile adults may be found in Britain at all times of the year, with the greatest numbers occurring between the months of May and August. Gametes are released into the water column where fertilisation takes place and in *circa* 16 hours free-swimming, planktotrophic trochophore larvae have developed.

Segrove (1941), described the laboratory culture and larval development of the closely allied species, *P. triqueter*, in some detail, covering development from the egg to metamorphosis. Segrove's study also included detailed microscopical investigations of the larval stages, metamorphosis and settlement. Several metamorphic stages were described but these stages were disputed by Føyn & Gjøen (1954) who considered them to be abnormal. Segrove's work was largely repeated and confirmed

by Moat (1985) as part of his doctoral study but doubt still remains regarding the disputed metamorphic stages.

Pomatoceros lamarckii larvae are not difficult to culture. Ova and sperm can be obtained at any time of year, late spring to summer being the optimum period for obtaining the gametes. Such ease of culture makes *Pomatoceros lamarckii* a very useful animal for research purposes. Segrove (1941), Føyn & Gjøen (1954) and Moat (1985) all deal with the laboratory culture of *P. triqueter* larvae, their methods being rather similar. The culture methods used in the course of the present study differ somewhat in detail from those of previous workers and it is hoped to show that advantages are to be gained from these differences in technique.

MATERIALS AND METHODS

Animals to be used for culture purposes were collected from the Anglesey shore of the Menai Strait in the vicinity of the Telford road bridge. For subsequent ease of handling it was found best to collect small, flat stones, *circa* 100mm. maximum diameter, on which were large, adult *P. lamarckii.* These stones were transferred to a tank of running seawater in the laboratory aquarium, where they were kept until required.

Culture procedure

Selected rocks were first vigorously scrubbed in seawater to remove any loose material and unwanted organisms before being placed in a shallow container and covered with seawater obtained from the laboratory running seawater supply. 25ml. glass beakers each containing approx-

imately 2ml. fine filtered (0.2μm. final filter) and ultra-violet irradiated seawater (FFSW) were used to hold collected gametes.

In order to obtain gametes it was first necessary to expose the abdominal segments of an animal. With the aid of a dissecting microscope the older and more fragile posterior end of the tube was carefully broken away, gradually working towards the anterior end. A strong instrument is required to prise away the pieces of tube which are often very hard and firmly attached to the substratum. A carpenter's bradawl, which has a short, chisel-edged blade and a rounded handle which fits nicely into the palm of the hand, was found to be ideal for this purpose. The disturbance created by removing part of the tube normally causes the animal to move forward until it reaches the feeding position with branchial crowns extended.

When sufficient tube had been removed any debris was flushed away from the broken end with a jet of water from a Pasteur pipette and the animal was gently eased backwards by pressing on the operculum with a wire of suitable diameter to enable it to pass down the tube. Tinned copper wire with a diameter of 0.5mm. was found to be ideal as it is very malleable and can easily be curved to conform with bends in the tube. The colour of the emerging abdominal segments indicates the sex of ripe animals. In general, fertile males have creamy-white abdominal segments whereas those of the fertile females are a bright red/pink, but some fertile males have quite dark abdominal segments and those of fertile females can vary between red/pink and a deep purple. When the animal was eased backwards gametes would almost immediately be seen to emerge from the gonoducts along the ventral groove (very ripe animals would start to release gametes during the process of shortening the tube) and these

gametes were collected with a Pasteur pipette and placed into the 25ml. beakers. Male and female gametes were kept separate at this stage. Gametes from several males were collected in one beaker but it was found best, if plentiful, to place ova from different females into separate beakers. This procedure allowed ova to be quickly fertilised if spermatozoa were already available and increased the chances of a successful fertilisation.

When sufficient ova and sperm had been collected the sperm sample was diluted as necessary with FFSW and gently stirred in order to distribute the sperm evenly. 2 ml. of the sperm dilution were added to each beaker of ova, stirred gently, covered and allowed to stand for ten minutes in order to allow fertilisation to take place. The water was then changed by carefully pipetting off and replacing with FFSW. The beakers with the fertilised ova were placed in a controlled environment (CE) cabinet at 18°C with a 12:12h. light:dark regime. After one hour the water was again changed and the beakers left in the CE cabinet overnight to allow embryonic development.

The number of larvae obtained after a fertilisation determined the size of the culture vessel in which they would be kept. Small numbers (<1000) of developing larvae were cultured in FFSW in 250ml. crystallising dishes and larger numbers in one litre beakers. All culture vessels were covered to reduce evaporation and maintained in the CE cabinet. Aeration was found to be unnecessary. Larvae were fed with a 1:1 mixture of two unicellular algae; *Tetraselmis chui* and *Rhinomonas reticulata*, added to the water on day one of development at a rate of 200 cells μl^{-1}. Algal counts were made with a Coulter counter. Culture water was changed every five days and fresh algae added. This procedure was found to be preferable to simply adding more algae when necessary as it ensured that there was little

build-up of toxic wastes and salinity did not increase beyond tolerance levels. Moat (1985) found that *Pomatoceros triqueter* could tolerate salinities as low as 18‰ but did not investigate the effects of increased salinity. Specific experiments to determine increased salinity tolerance of *P. lamarckii* larvae were not carried out during the present study but it was noted that at 40‰ salinity larvae developed normally but at 60‰ salinity they became deformed and died. Typically this deformity took the form of an evagination of the gut through the anus (Fig. 1).

Water changing was accomplished by using a simple tube with 90µm. plankton net glued to one end to act as a filter. Care was necessary to ensure that the larvae were not damaged during the filtration process. Ensuring that the filter was immersed in seawater while the water containing the larvae was gently poured through prevented the larvae being crushed against the mesh. The larvae were then transferred to clean FFSW by inverting the filter and gently pouring FFSW through the plankton net.

Microscopy

Light microscopy was carried out with a Leitz Dialux compound microscope and a Wild M3Z dissecting microscope. Photographs were taken with a Leitz Orthoplan photomicroscope using 35mm. film.

Specimens were prepared for scanning electron microscopy (SEM) by fixing in 2.5% glutaraldehyde in seawater, rinsing in distilled water and then dehydrating through an ascending ethanol series and finally through acetone before critical point drying from liquid carbon dioxide in a Polaron E3000 critical point drying apparatus. Small specimens such as larvae are easily lost during preparation for SEM. To minimise such losses specimens were always processed in a small, acetone resistant, plastic tube

(8mm. diameter x 14mm. long) with removable end caps. These end caps had their centre sections removed and 45µm. mesh plankton net fitted. The tube containing the specimens to be processed was placed upright in a watchglass containing FFSW. The water was then removed from the watchglass using a Pasteur pipette and replaced by glutaraldehyde which was put into the top of the tube. This procedure was repeated for each stage of the preparation (Fig. 2). After going through the acetone the tube, now closed at both ends, could be placed in the boat of the critical point dryer.

A single eyelash glued to the end of a cocktail stick with a dab of 'Tippex' erasing fluid was found to be the best tool with which to pick up individual specimens following critical point drying and to attach them to aluminium SEM stubs by means of double-sided 'Sellotape'. Static electricity was a problem during this procedure and most specimen losses occurred at this stage. This problem could sometimes be alleviated by the use of an anti-static gun. Finally, specimens were coated with gold using a Polaron E5000 sputtering system before viewing in a Cambridge Stereoscan S120 scanning electron microscope operated at 10Kv.

When studying *Pomatoceros* species larval development it is not always easy to determine whether a single eyespot is situated on the left or right side due to the transparency of the larvae. In order to make an accurate determination, measurement of the location of eyespots was related to the ventrally situated mouth and observations were commenced with the compound microscope out of focus above the larva being viewed. The microscope was then gradually brought into focus on the upper surface of the larva, then the interior and finally the lower surface before going out of focus on the lower side. This method accurately determined the

relationship of the eyespot to the mouth and other organs and thus it was possible to judge the position of the eyespot with some certainty. Any larvae in which the position of the eyespot remained ambiguous were not included in the results.

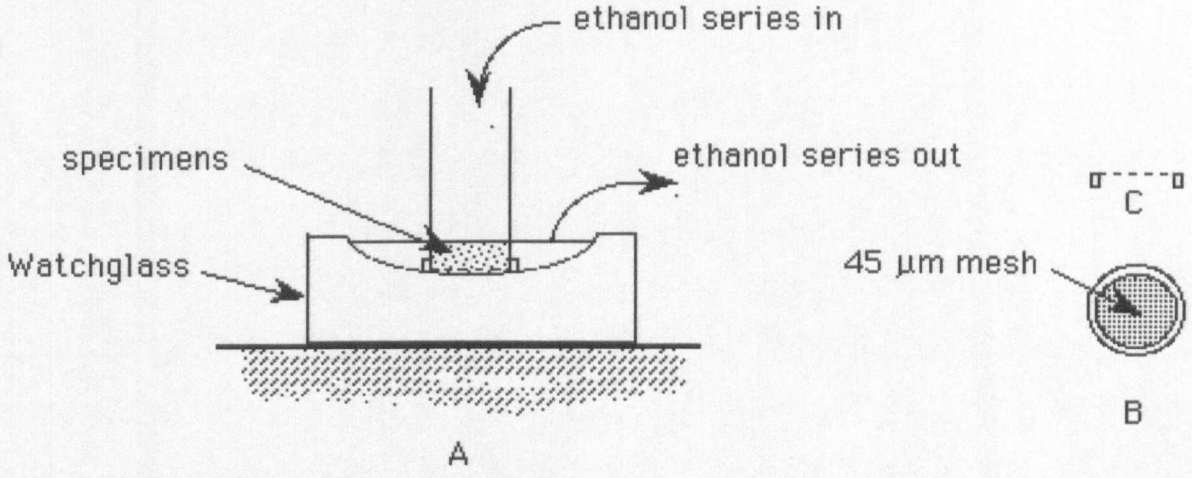


Fig. 2: The system for taking small specimens through an alcohol series. **A** = apparatus. **B** = end-cap, plan view. **C** = end-cap, elevation.

RESULTS AND DISCUSSION

Cragg (1939) observed that large specimens of *Pomatoceros triqueter* were usually female and the smaller ones male. He hypothesised that this size difference was due either to a size difference between the sexes or to a sex reversal. Føyn & Gjøen (1954) discovered that *P. triqueter* was in fact protandrous, the animals being first male and later becoming female. It follows, therefore, that the older and thus larger animals should be female. Ekaratne (1982), however, noted that females were not necessarily larger than males. Observations made during the course of the present study

support those made by Ekaratne. It cannot be stated with any certainty that a large animal will be a mature female.

When obtaining gametes previous workers (Segrove, 1941; Føyn & Gjøen, 1954; Moat, 1985) removed animals completely from their tubes by breaking open the tubes. (During the present study it was found that in a great number of cases this method resulted in damage to the animals.) The freed animals were transferred to seawater in beakers to produce gametes. Føyn & Gjøen (1954) severed the animals immediately below the thoracic segments and placed only the abdominal segments in beakers. Use of animals treated in this way and of damaged animals results in the release of body contents such as digestive enzymes into the culture. While there is no evidence that this release of enzymes has any adverse effect on fertilisation, it is obviously desirable to keep the culture as contaminant-free as possible. None of the animals used to obtain gametes by the above methods would survive in any viable form. *Pomatoceros lamarckii,* once removed from its tube is, in normal circumstances, unable to form a new tube and thus even the few animals which do not sustain body damage are not equipped to survive if returned to the wild.

Using the method employed in the present study, sexual maturity can quickly be determined, and immature or unwanted animals can be left to recover with minimal tube damage. Damage to animals cannot be entirely eliminated in situations of gregarious settlement where dense masses of tubes are intertwined and often attached to each other. However, in the majority of cases, this method of obtaining gametes results in no harm to the animals, which are not removed from their tubes, and, in a few days, are able to build a calcareous end-plate into the tube (Fig. 3). This end-plate prevents the ingress of predators and the animal can then be returned

to the wild. There are two further advantages to the present method. Should newly formed tube be required for experimental purposes it is readily available as animals with damaged tubes, in addition to building an end block, soon start to increase their tubes to a comfortable length. Secondly, animals can easily be extracted undamaged from their tubes by the described method if required for other experimental purposes.

Ideally fertilisation should take place as quickly as possible when preparing a culture but it is not always possible to obtain males and females in sequence. Føyn & Gjøen (1954) observed that sperm are active for up to a day but for good results should be used within the first half hour; ova should be used immediately. The results of the present study indicate that both sperm and ova are viable for around an hour. Beyond this time the outcome of a fertilisation is likely to be unsatisfactory with few, if any, larvae developing. By keeping batches of ova from separate females in separate beakers successful fertilisation is optimised, particularly if sperm are obtained before ova, by fertilising each batch of ova as it is obtained. Fertilisation is very rapid, the majority of ova probably being fertilised within 30 seconds of the sperm being added. The recommended time for fertilisation to take place (10 minutes) is based on a purely practical consideration. After gentle stirring it takes approximately 10 minutes for the eggs to settle out on the bottom of the beaker and until the eggs have settled it is not possible to change the water without risking considerable loss of zygotes.

Recent work (Cohen *et al.*, 1992) has drawn attention to the effectiveness of the spermatozoa of *Pomatoceros* species. It was found that only a small proportion (1:30 - 1:40) of sperm were capable of fertilisation and it was suggested that "Only a tiny fraction of the spermatozoa can

fertilise under these experimental conditions, due either to real, permanent heterogeneity in gametic potential or to the fact that only this tiny fraction is precisely 'ripe' ". As the animals had been induced to produce gametes it is quite possible that both sperm and ova were, in fact, not quite viable at the time of collection. However, these findings would appear to support the practice of collecting sperm from several animals in one beaker, as it at least increases the chance of providing viable sperm for fertilisation.

Pomatoceros lamarckii produces typical 'primitive' sperm with a short, bullet-shaped head-piece, 0.25µm. long by 0.16µm. wide, and a very long tail (Fig. 4). Compared to the advanced filiform sperm as found in the barnacle *Balanus balanoides* (Munn & Barnes, 1970) they are relatively slow moving and often remain stationary for short periods of time. The indentation which is often to be seen in the ova, presumably caused by contact with other ova within the coelom, noted by Cragg (1939) and Segrove (1941), can also clearly be seen in Fig. 4.

Cleavage is spiral. 60 minutes after fertilisation polarisation of the nucleus can be seen and the first cleavage of the zygote occurs in *circa* 90 minutes (Fig. 5), which concurs with the observations of Cragg (1939). The second cleavage is between 120 and 150 minutes and the next cell division may be seen after about 180 minutes.

Larval development proceeds very much along the lines described by Segrove (1941) and the sizes of larvae at various stages of growth compare well with the measurements which he recorded (Graph 1). However, it is worth recording the fact that in any culture some larvae remain much smaller than the mean size at any time, e.g. at day twelve, when most larvae are about to metamorphose, mean length and width (at the prototroch) are *circa* 315µm. and 170µm. respectively but perfectly

Figure 1.

Light micrograph of a deformed, 8 - day old *Pomatoceros lamarckii* larva. The gut (g) is evaginated through the dorsally situated anus. Scale bar...50 μm.

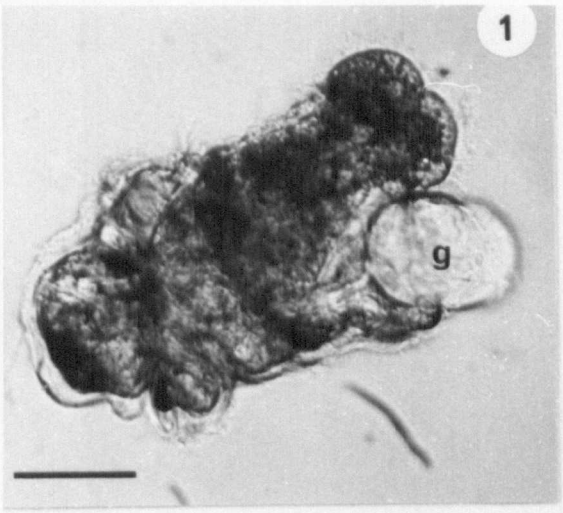
Figure 3.

Light micrograph of a *Pomatoceros lamarckii* calcareous end-plate sealing a glass tube. The rows of what appear to be holes are, in fact, sealed with clear mucus. Scale bar...500 μm.

Figure 4.

Scanning electron micrograph of a *Pomatoceros lamarckii* ovum with attached spermatozoa. Note the indentation, caused by close packing with other ova within the coelom. Scale bar...10 μm.

Inset: The sperm has a primitive type, bullet-shaped head which appears to have attached to the ovum by strands of cytoplasm. Scale bar...15 μm.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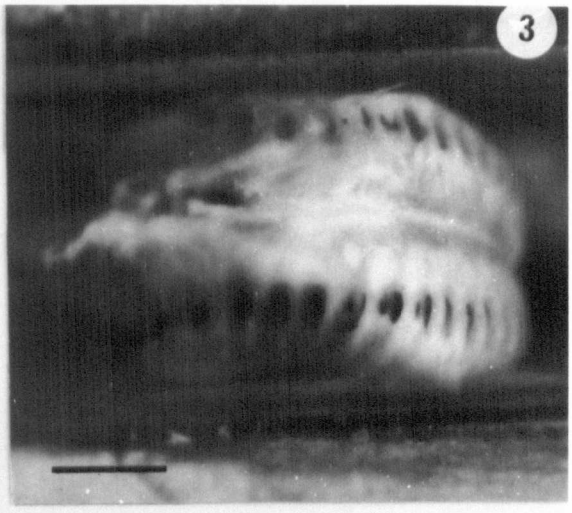
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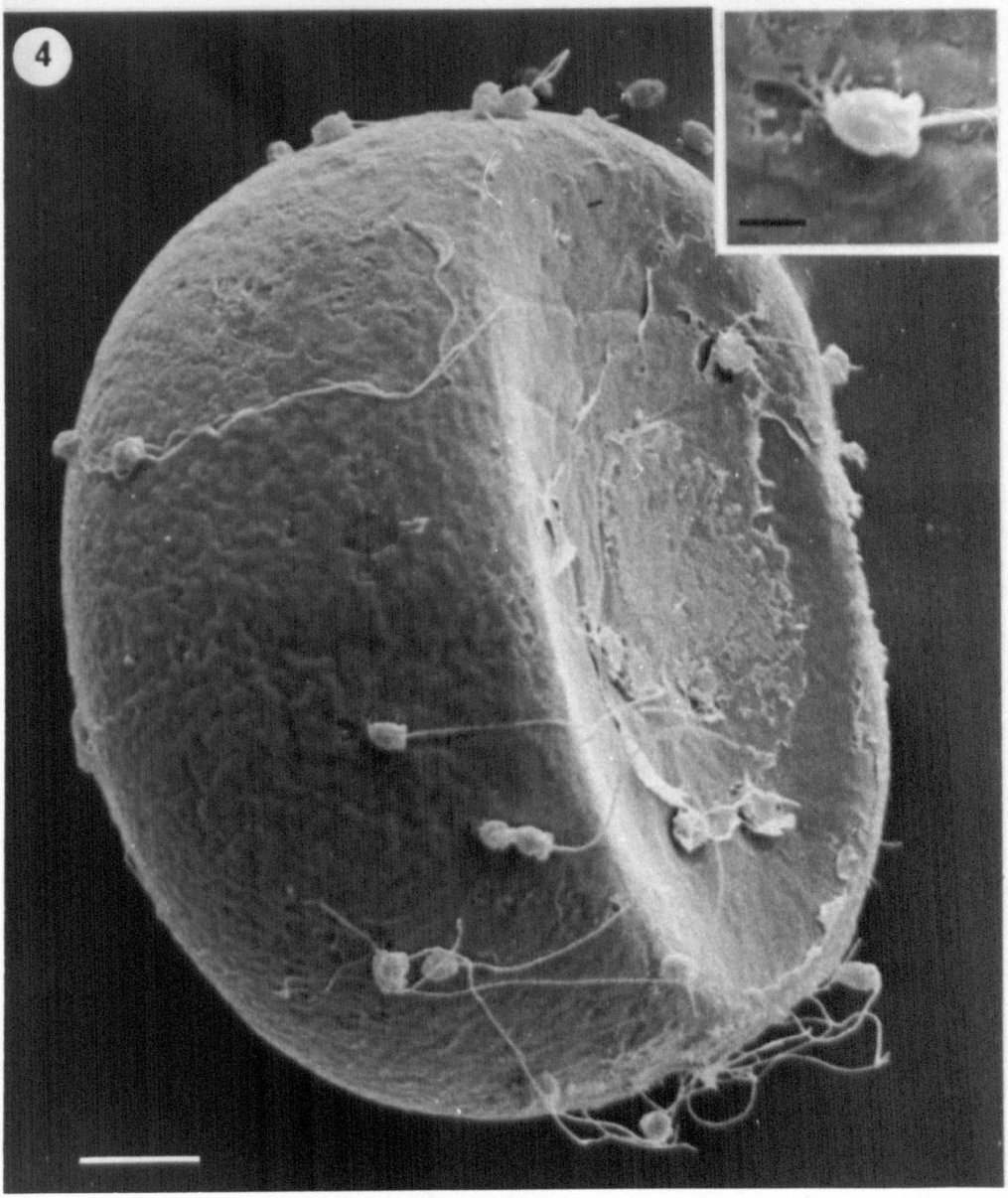
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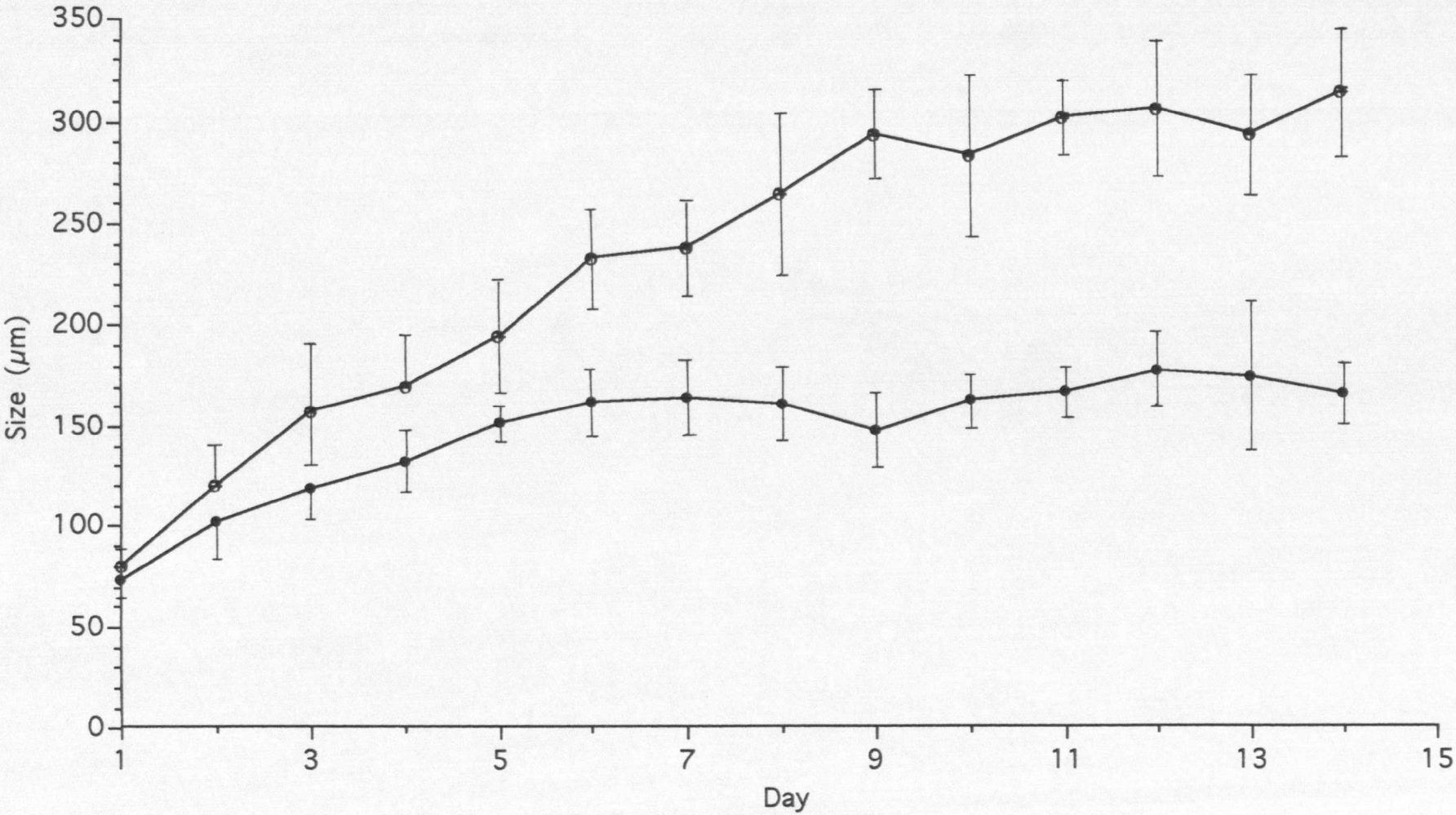


Graph 1: The growth of *Pomatoceros lamarckii* larvae from day 1 to day 14, plotted as mean lengths along the median line (o) and mean widths at the prototroch (•), excluding cilia, ± one SD (n = 10). Note the slight decrease in prototroch width at day nine with a subsequent increase as the rudiments of the collar start to develop.

formed larvae of around 200µm. long by 130µm. wide can still be found. Possibly these larvae have failed to secure an adequate share of the available food but as food is normally plentiful in larval cultures it is quite likely that this size discrepancy is a survival strategy. By having some larvae in a brood which grow at a slower rate than the norm, the total time spent in the water column by that brood is increased with a subsequent staggering of the settlement period. If all the larvae of a brood reached metamorphosis at the same time and no suitable settlement site was available, then all those larvae might perish.

Larvae become free-swimming at *circa* 16 hours and at this stage are little more than a rounded ball of cells with the prototroch to be seen as a median band of cilia. Interestingly, when viewed by SEM, the posterior surface of many of these larvae can be seen to be covered by a membrane (Fig. 6). The purpose and origin of this membrane are not known. Many of these early larvae appear to be loosely attached to the substratum and have to make an effort to break free. It is quite possible that the membrane is the remains of a mucous pad which forms this attachment. By day three the larvae have become fully formed trochophore larvae with the typical 'spinning top' shape and motion, prototroch and metatroch and a fully developed neurotroch on the ventral surface (Fig.7). The large anal vesicle which displaces the anus onto the dorsal surface is also present at this stage. At three days old the larvae develop one eyespot. Segrove (1941) stated that this eyespot was always the right hand one. While this order of development is predominantly the case, occasional larvae with initial development of the left eyespot have been observed during the present study.

Figure 5.

Light micrograph of fertilised *Pomatoceros lamarckii* ova at first and second cleavage stages of development (left & right respectively). Scale bar...70 μm.

Figure 6.

Scanning electron micrograph of a 1 - day old *Pomatoceros lamarckii* larva showing the prototroch (p) and a sheet of mucus (arrow) attached to the posterior part of the body. Scale bar...15 μm.

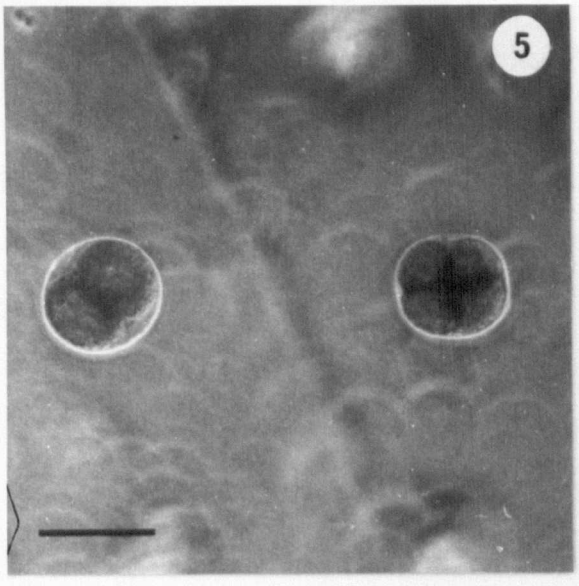
Figure 7.

Scanning electron micrograph of a 3 - day old *Pomatoceros lamarckii* larva. Prototroch (p), metatroch (m), neurotroch (n) and apical cilia (a) are all fully developed. Scale bar...15 μm.

Figure 8.

Scanning electron micrograph of a typical late metatrochophore larva at 15 days old. The prototroch (p) and metatroch (m) are reduced and a distinctive 'head' region (hr) has formed. The neurotroch (n) is still complete and the body is elongated. Scale bar...40 μm.

Inset: Light micrograph taken at the same stage of development. Eyes (e), stomach (s), and the beginning of the collar folds (cf) can be clearly seen. Scale bar...150 μm.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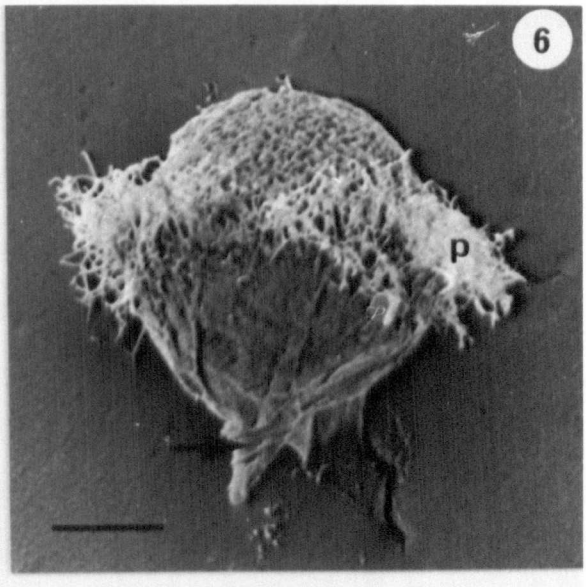
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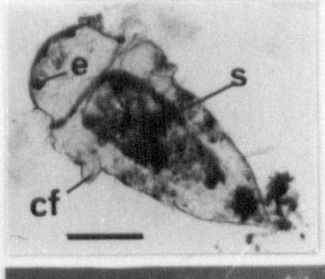
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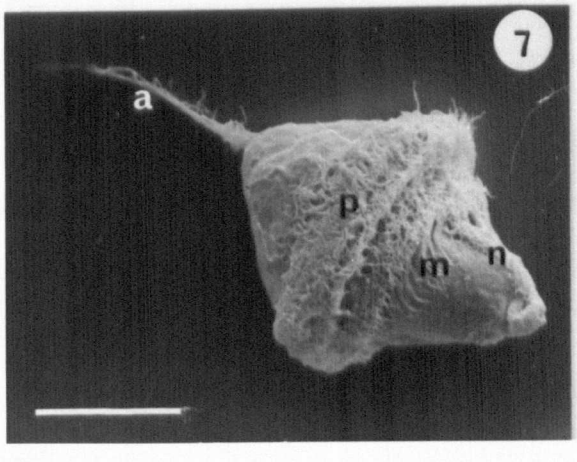
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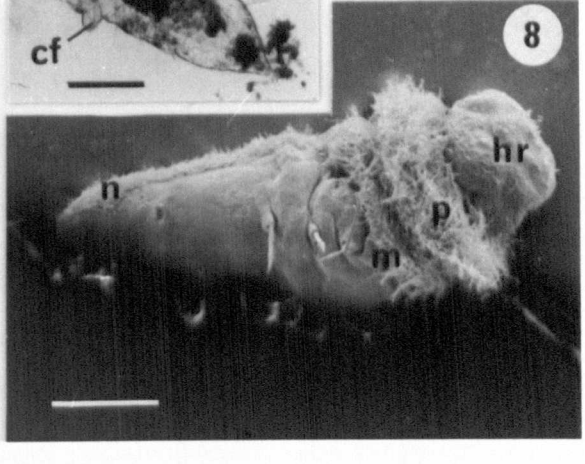
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Trochophore larvae often adopt a head-downward position with the apical cilia in contact with the substratum and will remain in this position for some seconds. It is unlikely that these larvae are sensing the surface with a view to settlement as this behaviour is frequently observed in animals which are only a few days old and is not consistent with the behaviour shown by larvae which are ready to settle. The more feasible explanation of this behaviour is that this position is an efficient feeding posture. The base of the culture vessel quickly becomes coated with a layer of algae and it would appear that larvae in the head-down position are feeding on these algae. When in this position it can be seen that a strong current is created which draws in many more algae than in normal swimming. These algae are unable to escape if swept into the feeding cilia around the mouth. When the larva finally swims away a small circular area of relatively clean glass, *circa* 500µm. diameter, is to be seen.

Larval development continues as described by Segrove (1941) until the larva has fully developed and is ready to settle. Given optimum culture conditions, this metatrochophore stage is normally attained in 9 -12 days. The larva is now much more worm-like (Fig. 8). There is some reduction in width at the prototroch as a distinct head area starts to appear (Graph 1) but it still retains the prototroch cilia and is still an active and fast swimmer. The mean speed of twelve day old larvae (n = 73) was found to be 1.03 mm. sec^{-1}.

At this point there is disagreement between the observations of Segrove (1941), Føyn & Gjøen (1954), and Moat (1985). Segrove (1941) gives a description and figures of several metamorphic stages prior to settlement and tube formation. The first of these figures (Fig. 9a) showed a larva which had lost the cilia of the prototroch and metatroch and also

part of the neurotroch. This stage was referred to as "the creeping stage" as the larvae, now unable to swim due to the loss of the prototroch and metatroch, were seen to creep about on their ventral surfaces, using the neurotroch cilia as their sole means of propulsion. Føyn & Gjøen (1954) considered such larvae to have become too old to settle and metamorphose.

Figures 9b & c illustrate the first stages of growth of the branchial crowns in larvae which had not settled and secreted a tube. Føyn & Gjøen (1954) attributed these forms to larvae which had settled, secreted tubes and then crept out of their tubes. They stated that, in their opinion, all the stages of metamorphosis as illustrated by Segrove (1941) in his figures 17 - 19 were the result of poor culture conditions, particularly the choice of food organism (a diatom; *Nitzschia* species), leading to many malformed larvae. They considered good culture conditions to be those which resulted in almost 100% successful settlement. It is worth noting at this point that the rate of settlement which Moat (1985) obtained in the laboratory seldom exceeded 30%. This rate of settlement reflects not poor culture conditions but the fact that Moat was attempting to obtain settlement on experimental surfaces, whereas Føyn & Gjøen (1954) were aiming for *Pomatoceros triqueter* adults from larvae which settled in the culture vessel. A settlement above 20% on an experimental surface in the present study was considered to be a good settlement. The mean settlement from 47 experiments was 35.6%. The highest settlement recorded being an isolated 90.1%.

Moat (1985) failed to identify the later larval stages as illustrated in Figures 9b & c, the latest motile larval stage identified being that of the creeping stage larva which, he stated; "....do occur immediately prior to metamorphosis and settlement, and appear to confirm Segrove's (1941)

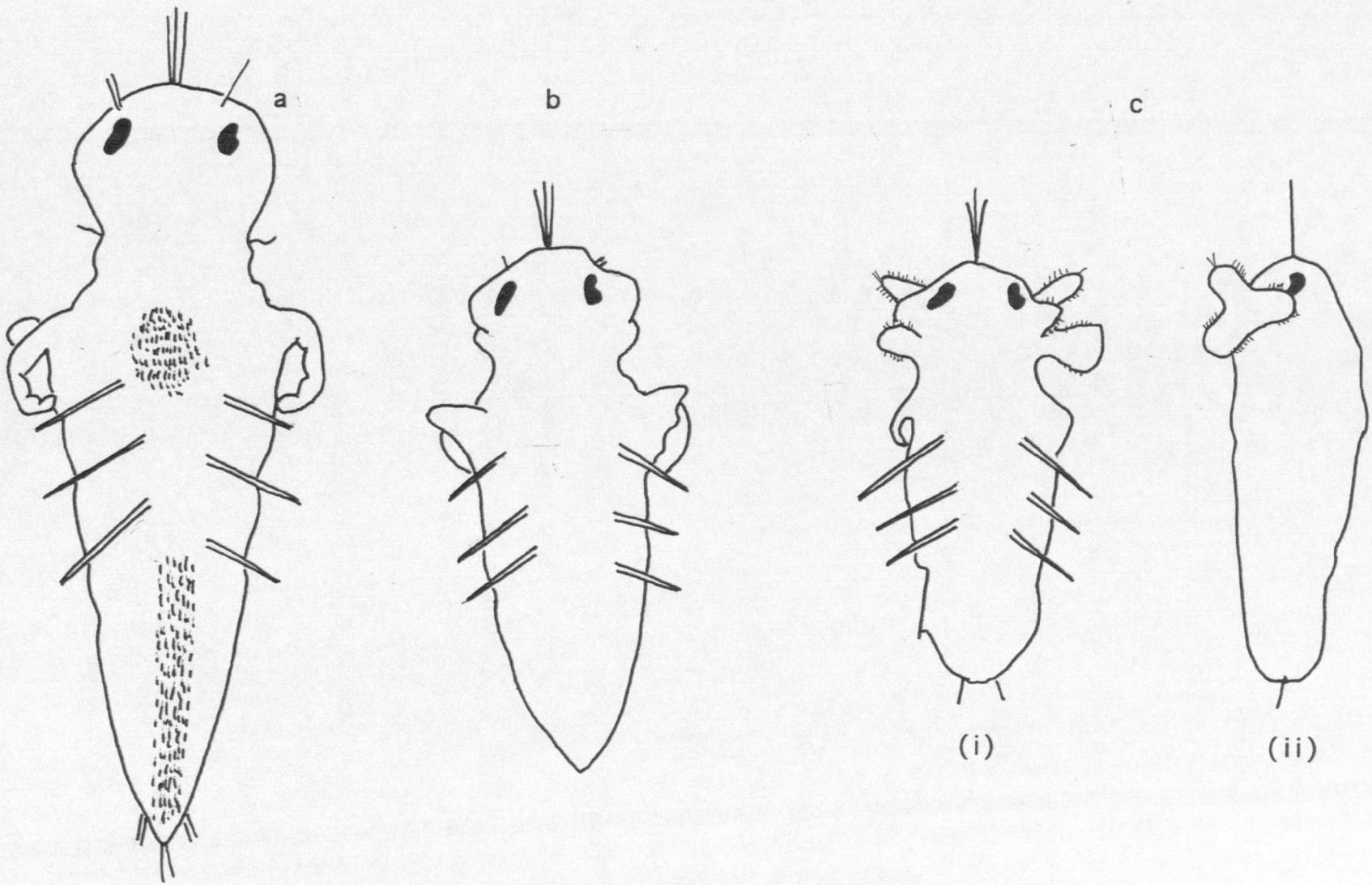


Fig. 9: Metamorphosis of *Pomatoceros triqueter* larvae. After Segrove (1941), figures 17 - 19 (all x375). **a**: Ventral view. **b**: Dorsal view, later stage. **c**: Advanced stage; **(i)** ventral view, **(ii)** lateral view.

view that the course of metamorphosis in *Pomatoceros* is initiated by the regression of the locomotory apparatus of the prototroch and metatroch cilia".

Examples of all the stages of larval development described by Segrove (1941) were observed during the present study and it is now possible to describe more fully what is actually taking place. In the first instance there can now be no doubt that *Pomatoceros* species larvae, given suitable stimuli, settle readily at the metatrochophore stage when they are still very active and have all locomotory cilia intact. Such settlement has been observed on many occasions during this study and has also been recorded on videotape by Mr. David Spears of Science Pictures Ltd. during a visit to the Marine Science laboratories at Menai Bridge. The minimum time recorded from larvae first becoming motile to settlement was eight days but settlement usually occurred between days 9-12. The settlement behaviour of *Pomatoceros lamarckii* larvae is described in detail in chapter 5.

If larvae fail to find attractive substrata on which to settle they then start to lose the prototroch and metatroch cilia and swimming becomes erratic due to uneven loss of these ciliated bands. Finally the larvae reach the creeping stage as described by Segrove (1941) with a narrow 'neck' in the area previously occupied by the prototroch, a distinct head region and the conspicuous bulges of the collar folds (Fig. 9a.). Some of these creeping stage larvae are still capable of settling and forming a tube if they are presented with an attractive substratum onto which they are able to crawl. The word 'some' is used advisedly and reflects one of the peculiarities of marine invertebrate larval settlement. As mentioned earlier, even on an obviously attractive experimental surface, 100%

settlement is never attained. Larvae which appear to be normal in every way, do not display any signs of attraction to a surface upon which other larvae, from the same batch and reared in the same culture vessel, settle readily. It would appear, therefore, that if some creeping stage larvae are able to settle, this stage of development is an age related phenomenon and the larvae are not malformed.

The larvae which Segrove (1941) described as later stages of metamorphosis (Figs. 9b & c.) have frequently been observed during the course of the present study. Occasionally larvae which have become tail fixed but have neither been able to escape nor to attach and form a tube develop in this way. No evidence has been found to support the view held by Føyn & Gjøen (1954) that these larvae were those which had settled, secreted a tube and then left it. However, there can be little doubt that these larvae are in some way abnormal and do not represent genuine stages of metamorphosis. The normal course of metamorphosis as observed in this study is: metatrochophore ➨ settlement ➨ tube formation and loss of locomotory cilia ➨ formation of branchial crowns ➨ juvenile. Most of the larvae which do not settle when presented with an attractive surface quickly die and decompose while still at the metatrochophore stage. It is probable that some creeping stage larvae, if unable to settle, do develop as described by Segrove (1941) but this development has not been directly observed. What can be stated with some certainty is that these abnormal larvae are not viable. They have not been observed to produce a tube and do not develop as those larvae which have settled normally and produced a tube. The most likely cause of this aberrant development is that the mucus and/or calcium secreting glands have failed to function resulting in an

inability to form a tube. Eventually these abnormal larvae die and decompose without having attained the morphology of a juvenile worm.

Chapter 4

Reactions of *Pomatoceros lamarckii* Larvae to Light

INTRODUCTION

Pomatoceros lamarckii (Polychaeta: Sabellida: Serpulidae) is a sedentary serpulid worm of the littoral zone and its distinctive, keeled, calcareous tubes are common on rocky shores around the British Isles. These tubes are often to be found in profusion on rocks, stones and the shells of other marine invertebrates.

Very little attention has been paid to the light reactions of *P. lamarckii* larvae. Segrove (1941) noted that the larvae of *P. triqueter* were photopositive. Moat (1985) found *P. triqueter* larvae to be strongly photopositive by days 4 - 6 of development but stated that this positive phototaxis diminished with age, the larvae eventually becoming photonegative prior to settlement. Klöckner (1976) also found that the late trochophores became photonegative. It should be noted that, in all cases, these observations were of larval behaviour in culture vessels and not the results of experiments specifically designed to determine the light responses of *P. triqueter*.

No other original references to the light responses of *Pomatoceros* species have been found but the light response of the larvae of other serpulids has been investigated: e.g. Young & Chia (1982) found that

Serpula vermicularis was, when exposed to horizontally directed light, briefly positively phototaxic before becoming negatively phototaxic. The time taken for the change from positive to negative phototaxis was an inverse function of the light intensity. Metatrochophores (defined by Young & Chia as those larvae with two eyespots) were found to be continuously and strongly negatively phototaxic. *Spirobranchus giganteus* was found by Marsden (1984, 1986) to display positive phototaxis initially and became photoneutral as a late metatrochophore. *Galeolaria caespitosa* initially displayed negative phototaxis and became photopositive at late metatrochophore stage (Marsden 1988). Marsden (1990) showed that *Spirobranchus polycerus* was quite variable in its response to light. Thorson (1964) collated data on 141 species of pelagic larvae, 82% of which were found to respond positively to light and migrate to surface layers. 12% seemed indifferent to light while 6% responded negatively to light and continued to do so during their whole pelagic life. In later stages most photopositive larvae became photonegative but larvae of many intertidal species remained photopositive until they stopped swimming. He stated that *Pomatoceros triqueter* was photopositive throughout but appeared to base this observation solely on the work of Segrove (1941).

The terminology used in the measurement of light can be a source of some confusion as the units used by researchers to quantify the amount of light reaching an organism vary widely, e.g. foot candles, lux, quanta m^{-2} sec^{-1}, microeinsteins (μE) m^{-2} sec^{-1}, Watts (W) m^{-2}. As will be seen, these units are not always precisely inter-convertible which leads to difficulty in drawing comparisons between the works of various authors. Lüning (1981) gives a comprehensive account of light measurement and terminology for biologists and the following resumé is based on this work.

'Old' measurements - lux & foot candles - are photometric measurements of illuminance, i.e. the range of light in the visible spectrum, but the range of wavelengths utilised by other organisms can be far broader than that detected by the human eye; e.g. plants are able to utilise a range from 300 - 800 nm. Therefore these photometric measurements are not always appropriate and radiometric measurements may be required. The plant biologist in particular is concerned with irradiance - the radiant energy incident upon unit area in unit time. The only permitted SI units for measurement of irradiance are: $W\ m^{-2} = J\ m^{-2}\ sec^{-1}$.

One logical approach to the measurement of irradiance is to measure the number of quanta (quanta = photons in the visible range) per unit area per unit time since the photochemical reactions within a plant depend on the number of quanta absorbed, not their energy content. Neither term nor unit has been prescribed so far for this type of measurement. Commonly used terms are: photon irradiance, quantum irradiance, quanta irradiance, photon flux density, photon fluence rate, photon flux.

With regard to units of irradiance, many authors now favour $\mu E\ cm^{-2}\ sec^{-1}$ or quanta $cm^{-2}\ sec^{-1}$. These units are easily inter-convertible:

$$1\mu E\ cm^{-2}\ sec^{-1} = 6.02 \times 10^{17}\ \text{quanta}\ cm^{-2}\ sec^{-1}.$$

As the microeinstein is not an SI unit some authors prefer to use the 'mol' instead. This use presents no problems as $1\ \mu mol\ cm^{-2}\ sec^{-1} = 1\mu E\ cm^{-2}\ sec^{-1}$.

Strictly speaking, one cannot make a direct comparison between units of illuminance and irradiance but, when wishing to convert the older

measurements of illuminance to irradiance for the purpose of comparison between publications, the following approximation is a useful guide:

$$1 \text{ W m}^{-2} = 5\mu\text{E m}^{-2} \text{ sec}^{-1} = 250 \text{ Lux.}$$

In the present work, which has both zoological and botanical aspects, light values are presented in terms of irradiance. The units of irradiance used are quanta cm^{-2} sec^{-1} but references to light values made by other workers are given in the units used by those particular workers.

MATERIALS AND METHODS

Stones holding colonies of *Pomatoceros lamarckii* were collected from the Anglesey shore of the Menai Strait in the vicinity of the Telford road bridge. These stones were transferred to a tank of running seawater in the laboratory aquarium where they were kept until required.

Gametes were obtained by breaking pieces off the posterior ends of large *P. lamarckii* tubes until the tubes were sufficiently short to allow the animal within to be pushed gently back by pressing on the operculum with a wire of suitable diameter. As the abdominal segments emerged from the posterior end of the tube mature gametes would be released from the gonoducts. These gametes were collected and placed into 25ml. glass beakers each containing a little fine filtered and ultra-violet irradiated seawater (FFSW). When sufficient ova had been obtained, the sperm suspension was added to the beaker containing the ova, gently stirred and the beaker allowed to stand for ten minutes for fertilisation to take place. The water was then changed by pipetting off and replacing with fresh

FFSW before covering the beaker containing the zygotes and placing it in a controlled environment (CE) cabinet at 18°C to allow the larvae to develop.

Pomatoceros species larvae become free-swimming in *circa* 16 hours after fertilisation. The fertilisation procedure was therefore normally arranged so that the larvae would develop overnight. The day on which the larvae became free-swimming was considered to be day one for experimental purposes. Small numbers of developing larvae were cultured in FFSW in 250ml. crystallising dishes and larger numbers in one litre beakers. These beakers were covered to prevent evaporation and placed in a CE cabinet at 18°C with a 12:12 h. light:dark regime. Aeration was found to be unnecessary. Larvae were fed with a 1:1 mixture of two unicellular algae; *Tetraselmis chui* and *Rhinomonas reticulata.* This mixture was added to the water on day one at a rate of 200 cells μl^{-1}. Culture water was changed every five days and fresh algae added.

Each light experiment was run over the ten day period which normally covers development from trochophore to settling stage metatrochophore larva. An experiment consisted of a number of tests for reaction to irradiance which were carried out in a test chamber. Whenever possible at least one test per day was carried out. In addition to these tests larval reactions to light were also assessed by observation of their behaviour in the culture vessel. These observations included reactions to a natural light source such as the light from a window and reactions to a point source of white light placed so that the beam was directed horizontally across the beaker.

The results were statistically analysed by Chi^2 (goodness of fit to a uniform distribution) analysis. A statistically uneven distribution between

light, mid and dark sectors of the test chamber was considered to be a photoresponse. Any other result was considered to be a neutral response. Strength of photoresponse was expressed as a percentage of the total number of larvae found in the light and dark sectors of the test chamber.

For the experimental assessment of light reaction a simple test chamber was constructed from a 150mm. x 50mm. x 25mm. block of ABS plastic into which was milled a channel 10mm. square in section and 120mm. in length. The channel was painted black with Indian ink and the open end of this channel was sealed with a glass plate, using clear silicone aquarium sealant as an adhesive. An aluminium cover was made and painted matt black to exclude all light except that coming through the glass plate (Fig. 1). The channel was marked out into 12 equal 10mm. sections and a point light source horizontally aligned with the glass plate so that the beam illuminated the channel when the ABS block was mounted on the stand of a binocular dissecting microscope. In use, this piece of apparatus was found to require modification (see Experimental Procedure below.) and was subsequently modified by slotting the block in two places to allow the insertion of 2mm. Perspex partitions which divided the channel into three equal areas. These areas are referred to as sectors 1, 2 and 3, sector 1 being that nearest to the light source and sector 3 that furthest away (Fig. 2).

Light microscopy was carried out with a Leitz Dialux compound microscope and a Wild M3Z dissecting microscope. The white light source was provided by a Volpi Intralux 250HL 250 watt fibre optic system in which the intensity of irradiance could be adjusted. It was also possible to insert into this light source coloured filters to change the wavelength of irradiance.

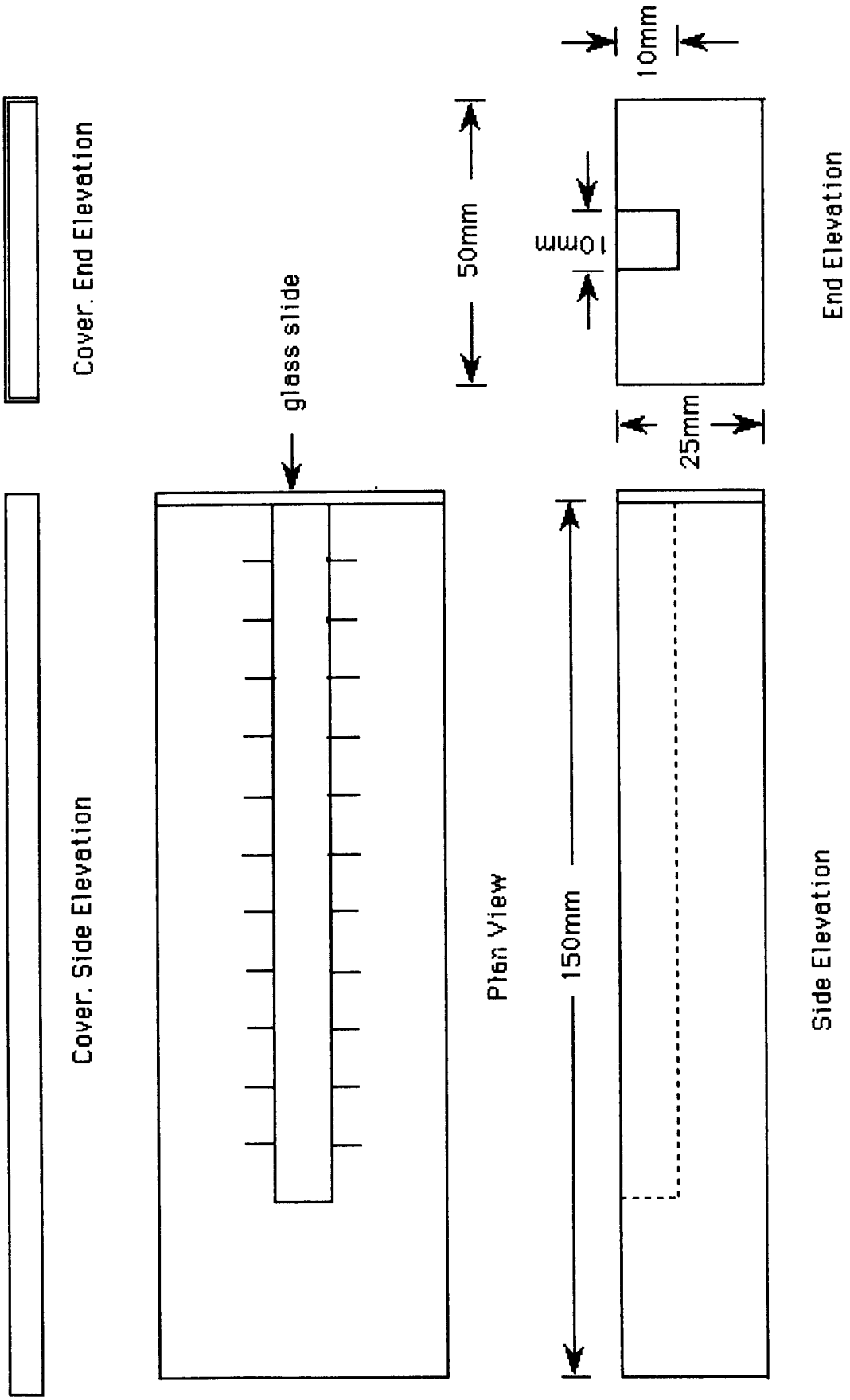


Fig. 1: The light chamber. Scale full size.

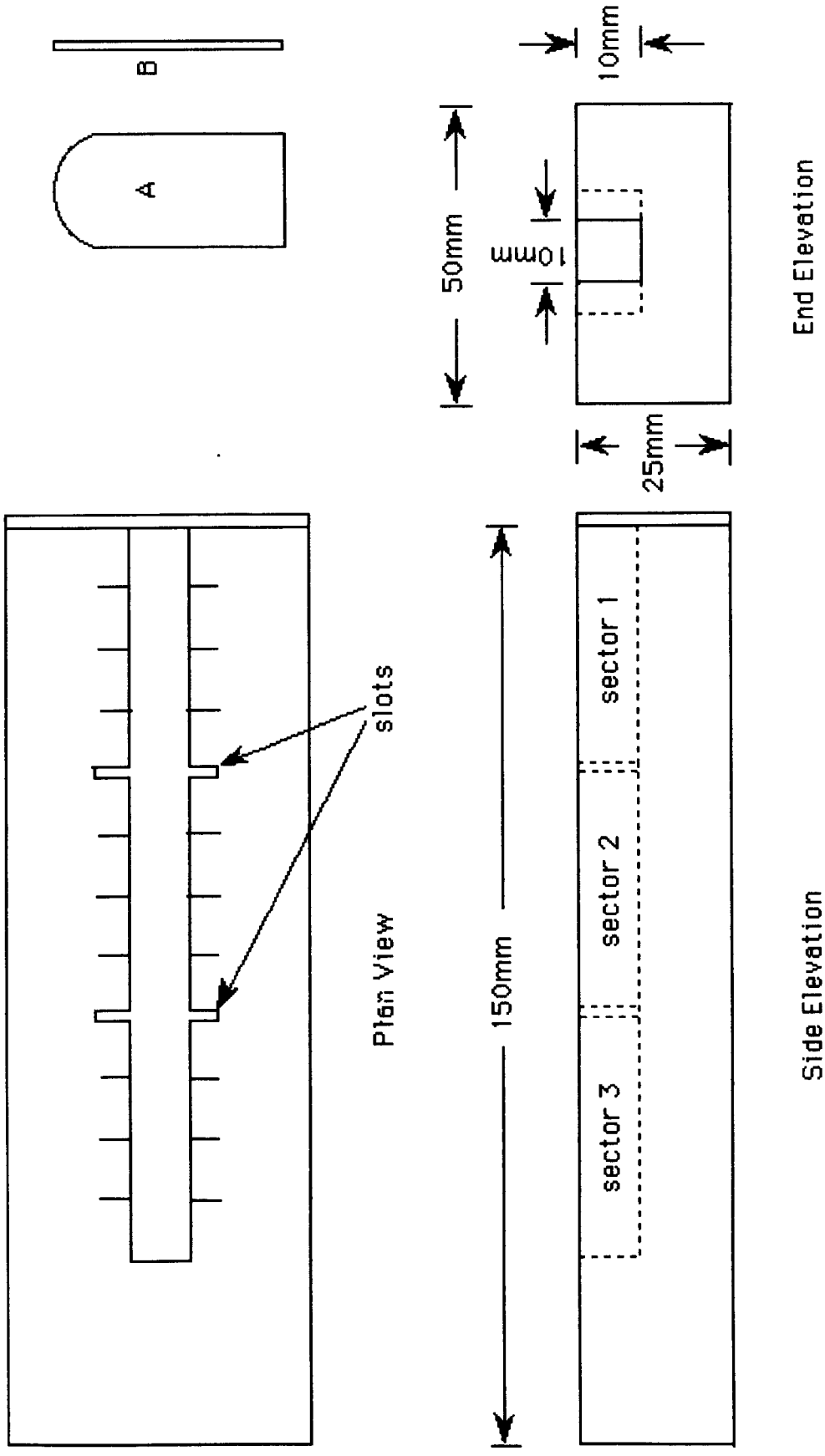


Fig. 2: The modified light chamber. Scale full size. A & B = Perspex partition; plan view and side elevation respectively.

Experimental Procedure

Prior to the commencement of an experiment, irradiance was adjusted to $1x10^{17}$ quanta cm^{-2} sec^{-1} measured at the glass plate using a Quantum Scalar irradiance meter, model QSL 100. The intensity of this irradiance reduced to 0.8×10^{16} quanta cm^{-2} sec^{-1} at the closed end of the channel. This second light measurement was made just above the channel as the irradiance meter probe was too large to enter the channel. To ensure that the temperature of the water in the experimental apparatus was initially the same as that of the larval culture vessel, a beaker of FFSW was kept in the CE cabinet containing the larvae to be tested. Using this FFSW, the channel in the test chamber was filled to one millimetre below the top and *circa* 110 larvae introduced. Counts of dark acclimatised larvae were made over a four day period in order to establish whether dark acclimatisation resulted in the desired random distribution of the larvae at the commencement of a test. The aluminium cover was placed on the test chamber and a black card placed between the light source and the glass in order to allow the larvae to dark acclimatise.

On completion of the fifteen minute dark acclimatisation period the light was switched on, the black card removed from the glass and the larvae exposed to the light for 15 minutes. At the end of this time the larvae in each 10mm. section of the channel were counted section by section, commencing at the end nearest to the light source by sliding the cover backwards so that initially only those larvae exposed to maximum light were exposed for counting. The limitations of this technique quickly became obvious:

- There was a tendency for the larvae to swim in and out of the area being counted.
- The test chamber had to be moved to get each successive 10mm area of the channel into the microscope field of view for counting. Even carried out extremely carefully this operation did tend to disturb the water and could conceivably alter the count.
- Larvae were not easy to see and count in the channel, particularly when using young trochophores.

It was noted that, in the main, larvae tended to aggregate close to the ends of the channel with fewer larvae in the centre. With these points in mind the test chamber was then modified as previously described. The Perspex partitions could quickly be inserted at the termination of each light period, sliding back the cover as previously described, from the light to the dark end of the channel. Once the partitions were in place the larvae could be counted accurately without fear of disturbance, or of larvae swimming into or out of the partitioned area. The larvae could be re-counted if necessary and it was thought that this modification significantly reduced experimental error.

When using the modified apparatus, the best method of counting the larvae was (following insertion of the Perspex partitions) to pipette the contents of each chamber into separate, labelled watchglasses. The larvae could then be counted from the watchglasses back into the culture vessel.

Sources of Error and Their Eradication

1 Larvae sticking in the pipette during transfers. This problem could lead to larvae from one chamber being transferred to the watchglass containing larvae from another chamber resulting in a false count. This problem is easily overcome by using dedicated pipettes for each chamber.

The exact number of larvae introduced to the test apparatus at the commencement of each experiment was unimportant as results were based on the number of larvae finally counted, not on the number originally introduced. 110 larvae were introduced which, allowing for some sticking in the pipette, meant that the actual number in the test apparatus should be around 100. A total count which differed appreciably from this number was an indication that an error in counting had probably occurred.

2. Numbers of larvae counted seldom corresponded to the number of larvae originally introduced into the test chamber. Tests made by transferring a known number of larvae in a watchglass into a second watchglass and then counting the transferred larvae, showed that even if the original watchglass was carefully flushed after the water containing the larvae was pipetted out some larvae still remained in the original watchglass. It was obvious that larvae tended to stick to the sides of the container in addition to the pipette.

In order to reduce the above sources of counting error each compartment of the test apparatus was flushed with FFSW immediately after the larvae had been pipetted off and this flushing water was in turn pipetted off and added to that containing the larvae in the appropriate watchglass. Each compartment was then refilled with FFSW. After the

larvae in the watchglasses had been counted the compartments of the light apparatus were examined and any further larvae found were added to the count. The adoption of this procedure resulted in counts which corresponded much more closely to the original number of larvae introduced.

3. Failure to spot all the larvae in a watchglass when counting.

4. Larvae escaping from the pipette when counting and thus being counted twice.

Errors three and four could be virtually eliminated by removing no more than five larvae from a watchglass at a time and watching the mouth of the pipette carefully for signs of emerging larvae until it was withdrawn from the water. Gently stirring the water dislodged larvae from the side of the watchglass so that they could more easily be seen. Occasional rotation of the watchglass would induce larvae to swim to a new location in response to the change in the direction of the light. Finally, after removal of the larvae, the watchglass was placed on one side and later checked a second time before discarding the water. This final check often revealed one or two larvae which, despite all precautions, had succeeded in avoiding detection.

The best procedure for counting can therefore be summarised as follows:

1. Pipette the contents of one chamber into a watchglass.
2. Rinse the chamber with FFSW and pipette the rinsing water into the same watchglass.
3. Refill the chamber with FFSW.

4. Repeat steps one to three for the other two chambers using dedicated pipettes.
5. Count the larvae in the watchglasses as described above.
6. Examine each chamber under the dissecting microscope and count any remaining larvae.
7. Make a final check for larvae before discarding the water in the watchglasses.

Tests for response to white, green, blue, and red light were carried out. Initially these tests were made consecutively but one series of tests took a working day to complete. It was considered that conditions could be quite different by the end of the day from those appertaining at the start of the test run, e.g. temperature, larval development and the response of larvae to being handled could all alter and could, in turn, affect the results. For this reason it was decided to limit tests to two per day. These tests usually consisted of exposure to white light and one other colour.

RESULTS

Dark Acclimatisation

There was no significant difference between the numbers of larvae in the three light sectors on any of the four test days (probability < 0.05). Therefore the larvae were assumed to be randomly distributed following a fifteen minute period of dark acclimatisation. (Table 1).

Table 1: The distribution of 1-4 day old *Pomatoceros lamarckii* larvae following a fifteen minute dark acclimatisation period.

Day	No. of larvae in sector 1.	No. of larvae in sector 2	No. of larvae in sector 3	X^2 value
1	32	26	34	1.13
2	21	28	31	1.98
3	37	28	23	3.43
4	20	29	36	4.54

Observed Response to Light

Table 2: The observed response to light of *Pomatoceros lamarckii* larvae from day one of development to day ten.

Day	Phototaxis	Observations
1	None	Larvae swim close to the surface but show no polarity.
2	Weakly negative	Less larvae at the surface.
3	Weakly negative	Mainly as day two but some batches showed a stronger negative tendency. Most larvae now have one eyespot.
4	Photonegative	Most larvae now swimming lower in the water column.
5	Quite variable	Some batches photonegative, others photopositive.
6	Mainly photonegative	Some larvae now with two eyespots.
7	Photonegative	Some larvae in any one batch photopositive but the majority photonegative. The majority of larvae now have two eyespots.
8	Variable.	Some batches very photonegative while others were very photopositive.
9	Strongly photopositive	Strongly photopositive at the surface but photonegative close to the substratum.
10	Strongly photopositive	As day nine.

As can be seen from Table 2, the observed response to light was quite varied from day to day and from one batch of larvae to another. It was also noticed that response to light could vary according to the position of the light source. When the light source was aligned with the water surface

positively phototaxic larvae were more strongly attracted than when it was aligned parallel to, and a few millimetres above, the base of the culture vessel. This difference in attraction was particularly marked in those larvae approaching metamorphosis at around 9-12 days old. At this stage two distinct groups were frequently observed; a surface group which appeared to be strongly positively phototaxic and a group swimming close to the substratum which appeared to be strongly negatively phototaxic.

Measured Light Responses

The results of experiment 1, which was carried out with the unmodified light chamber, are not included as there was too much possibility of error. However, these results did follow the same general pattern as the remainder of the experiments. No significant differences were found between the responses to white, red, green and blue light. As the white light results make up the bulk of the experimental work and therefore provide the largest sample for analysis, these results are given in Table 3 as being representative of the results as a whole.

Chi^2 analysis of the data showed that there was a significant difference between the numbers in the three sectors (probability < 0.05). It can be seen from Table 3 that on all days the majority of the larvae were to be found in the sector furthest from the light source, indicating that the larvae were negatively phototaxic from day 1 onwards.

Table 3: The distribution of 1-10 day old *Pomatoceros lamarckii* larvae following a fifteen minute period of exposure to white light. The figures in parenthesis indicate the total number of tests carried out per day.

Day	No. of larvae in sector 1.	No. of larvae in sector 2.	No. of larvae in sector 3	X^2 value
1.(4)	130	99	164	16.14
2.(4)	40	42	311	371.01
3.(4)	35	28	318	435.82
4.(3)	38	56	178	127.97
5.(2)	25	43	165	149.39
6.(3)	43	68	190	123.32
7.(3)	22	44	225	255.86
8.(4)	178	147	1060	1164.22
9.(4)	34	43	358	469.61
10.(3)	17	14	194	283.27

Table 4: The strength of negative photoresponse expressed as a percentage of the total number of larvae in sectors 1 & 3 of the test chamber.

Day	1	2	3	4	5	6	7	8	9	10
Strength of response	55.8	88.6	90.1	82.4	86.8	81.6	91.1	85.6	91.3	91.9

It can be seen from Table 4 that larvae were weakly negatively phototaxic on day one, becoming strongly negatively phototaxic from day two onward. An increase in photonegativity can be seen on days 3 and 7. This increase coincides with the development of one eyespot on day 3 and the second eyespot on day 7 and may occur as a result of this development.

DISCUSSION

A comparison of the results for observed and measured reactions to light (Tables 2, 3 & 4) reveals obvious discrepancies in the two sets of results, e.g. larvae observed in the culture vessel on days nine and ten showed every sign of being positively phototaxic, clustering around the area of a point light source aligned parallel with the water surface. When these same larvae were tested in the light chamber they proved to be strongly photonegative. These discrepancies led to an in-depth investigation of the experimental design in order to determine whether the discrepancies could be explained by experimental error. Some sources of

error were discovered and rectified. These sources of error and their solutions are given in Materials and Methods. Despite elimination of identified experimental errors there was still a marked difference between observed and tested results.

The observed results (Table 2) showed a great deal of variation. Other workers have also noted wide variability in observed larval responses to light and a number of factors which affect these responses have been identified. Temperature, pressure, salinity, light orientation and light intensity are all known to affect the photoresponse of marine invertebrate larvae (Ryland, 1960; Thorson, 1964; Crisp & Ritz, 1973; Forward, 1974). Thorson (1964) noted that the sign of phototaxis can change with light intensity, low intensity invoking a positive response and high intensity invoking a negative response. Crisp & Ritz (1973) stated that an increase in pressure induces positive phototaxis and a decrease in pressure induces negative phototaxis. Given such wide variability it is not surprising that previous workers have arrived at different conclusions as to the photoresponse of *Pomatoceros* larvae, but this variability does not explain why observed and measured responses should vary widely in larvae which have been observed and tested on the same day under similar conditions and light intensities. Logically, one would expect the measured response to be the most accurate but why should the observed larvae appear to be strongly photopositive?

In an attempt to resolve this question eight day old larvae showing strong positive phototaxis were pipetted from the group attracted to the light source and tested in the light chamber. As this sample was pipetted as a whole rather than counted, the number of larvae sampled was very high (1114) which explains the large number for Day 8 in Table 3. The result

of the test showed the larvae to be highly negatively phototaxic (85.6%). When clustering at the light source, the larvae were closely observed under the binocular microscope. It was seen that larvae did not stay in the light beam but sank below it or swam off to the side. This behaviour was observed on many occasions. When the light source was moved to the base of the culture vessel the larvae exhibited strong negative phototaxis. It would appear, therefore, that at any one time only a small proportion of the larvae in the culture vessel exhibit positive phototaxis, while the great majority are negatively phototaxic. It would also appear that the positive phototaxis is of relatively short duration but is real and not merely the result of some other factor such as convection flow. A sample taken from those larvae which were swimming towards the light source and tested in the light chamber for a period of five minutes showed these larvae to be 70% positively phototaxic. It is quite possible that a close approach to a light source provides the stimulus for a change in photoresponse. Sulkin (1990) says: "Each organism may have a characteristic stimulus threshold (time or intensity) above which it changes from indifference to either positive or negative (or from positive to negative)". It is also possible that contact with the wall of the culture vessel may trigger a change in response.

The above observations offer an adequate explanation for the apparent discrepancy between observed and measured results. On the basis of the results of the measured reactions to light it can therefore be said that *Pomatoceros lamarckii* larvae are predominantly photonegative during the first ten days of their life as free-swimming, planktotrophic larvae.

The other conclusion which can be drawn from the results is that young, one day old, *P. lamarckii* larvae are negatively geotaxic as the

majority are always to be found swimming at, or close to, the water surface despite being weakly (55.8%) negatively phototaxic.

Phototaxic response is of obvious importance to invertebrate larvae in the wild. Crisp & Ritz (1973) state that "light flux and gravity are the only orientating vectors available" (to invertebrate larvae). A planktonic larval stage serves the obvious function of dispersal of the species. Crisp (1974) gives three possible benefits of larval dispersal:

- Wider genetic exchange.
- Potential to colonise distant geographical areas.
- The ability to occupy an impermanent habitat.

The distance which can be travelled by planktonic larvae must be determined by the duration of the larval stage and the strength of the current. Crisp (1974) suggests that distances of 20 - 100 km. are attainable by larvae which remain in the plankton for 2 - 3 weeks and Scheltema (1986), using drift bottle data, established that teleplanic larvae are able to travel 2000 - 4000 km. over their 3 - 6 month pelagic period. A photoresponse or georesponse which results in larvae becoming established in regions of increased water flow is obviously of benefit in terms of larval dispersal. In the case of *Pomatoceros lamarckii* larvae it would appear that this establishment is initially achieved by a geonegative response but is possibly reinforced in later larval life by short periods of positive phototaxis. Thorson (1964) gives rapid escape from benthic filter feeders as another valid reason for a photopositive or geonegative response.

As sedentary polychaete larvae approach settlement and metamorphose one would expect them to become photonegative in order to explore the

substratum for suitable settlement sites. Crisp (1974) states that larvae which settle on substrata and eventually become sessile adults must become photonegative or geopositive. It does not appear to be advantageous for larvae to remain photonegative throughout most of their planktonic existence as this photonegativity would result in the larvae remaining low in the water column where water flow is less and consequently dispersal would also be less widespread than that of those larvae occupying the upper water column. There is some evidence that photonegative larvae 'explore' more than photopositive larvae (Ryland, 1960) but this exploration could be of no real advantage to trochophore larvae. Possibly larvae which remain low in the water column suffer less predation than those in the bulk of the plankton which is to be found high in the water column. A reasonably long planktonic existence would offset the disadvantage of slower dispersal and less predation would result in more larvae surviving to reach adulthood.

The results of the present work provide more information on the light responses of *Pomatoceros lamarckii* larvae than was previously available but further work remains to be done. All the present work was carried out with well fed larvae and it is possible that starved larvae may show a different response. There is evidence that satiated larvae of some other species sink to the bottom of the culture vessel, giving a false impression of photonegativity (G. Walker, pers. comm.). It is not thought that this is the case with *P. lamarckii* larvae as those larvae to be found exhibiting negative phototaxis at the bottom of the culture vessel respond immediately to a change in the direction of the light source by swimming away from it, indicating a true photonegative response. However, until experiments have been carried out with starved larvae, this possibility cannot be entirely

ruled out. Further tests need to be carried out with short duration exposures to a light source in order to ascertain whether the apparent initial positive photoresponse is genuine and, if so, the duration of this positive photoresponse needs to be established. Sulkin (1990) throws doubt on present methods of testing for photoresponse saying "Two experimental conditions .....are becoming increasingly difficult to justify: use of highly directional light and application of the light stimulus in a horizontal plane". The argument being that these conditions are not found in nature. Light intensity can also affect photoresponse (Thorson, 1964). Future experimental work should take these factors into account by varying both direction and intensity of irradiance in order to arrive at a true picture of the light response of *Pomatoceros lamarckii* to irradiance.

Chapter 5

The Settlement and Early Tube Formation of *Pomatoceros lamarckii*

INTRODUCTION

Pomatoceros lamarckii (Polychaeta: Sabellida: Serpulidae) is a sedentary serpulid worm of the littoral zone. It is to be found on a wide variety of hard substrata on rocky shores around the British Isles. The adult tube usually appears to be incomplete as no calcium carbonate can be seen on the base. This lack of calcium carbonate gives the impression that the tube ceases where it meets the substratum and that the substratum forms the base. Faouzi (1931) stated that the tube could not be complete owing to the gap which exists between the two lateral lobes of the collar on the dorsal side, but Thomas (1940) discovered that the tube, although normally apparently incomplete, is occasionally complete. Hedley (1956) identified the main source of organic material for tube construction as the ventro-lateral epithelial cells and described tube construction by the mature adult in some detail (Hedley, 1958). Neff (1968) working on the morphology of the calcium secreting glands of *P.caeruleus* found that the ventral shield epithelium contributes both organic matrix material and calcium for the formation of the tube.

Segrove (1941) gave a comprehensive account of the development and settlement of *P. triqueter* which remains the classical account of

Pomatoceros larval structure. However, settlement information was limited to a very brief description of where the larvae settled. No reference was made as to how they settled or what influenced the choice of settlement site. Føyn & Gjøen (1954) added a little more substance to the settlement observations. They noted that the settling larvae often initially attached by the posterior end. After settlement they secreted a fine, semi-transparent tube open at both ends. It was also noted that larvae tended to settle in small groups and favoured the junction of the base and sidewall of the glass settlement vessel.

Moat (1985), working with *P. triqueter*,[1] noted: "the remains of a mucoid attachment and mucoid tube at the posterior end of the first calcareous tube." Despite biofilming for two or three days, Moat (1985) failed to obtain laboratory settlement on a wide variety of substrata which had proved successful in the field. The only successful substratum for laboratory settlement was found to be old roofing slate. It was further noted that *P. triqueter* larvae preferentially settled on slates having resident members of their own species, but no mention was made of any settlement occurring on adult tubes. Moat's observations led him to believe that the anal vesicle, containing an adhesive mucous secretion of the anal mucous glands, may play a major role in permanent attachment.

Although little research into the settlement of *Pomatoceros* species larvae has been carried out, there is a considerable body of work available on the settlement of other marine invertebrate larvae. Some of this work relates to closely associated species of sedentary polychaetes and highlights settlement factors which many marine larvae hold in common. Knight-

[1] Moat specifically stated that, contrary to the findings of Zibrowius (1968), all specimens found in the intertidal zone were *P. triqueter* and that this species was used in all his work.

Jones (1951), working on the settlement of *Spirorbis* species, made several important discoveries. He was able to show that *Spirorbis* species larvae settled on glass or stone only if these substrata had algal films, that the majority settled on surfaces which bore their own species and that settlement took place earlier on surfaces which held previously settled individuals than on bare control surfaces. He also described three phases of larval searching behaviour. These phases were later described for other marine invertebrate larvae by Crisp (1974) who adopted the terminology of broad exploration, close exploration and inspection to distinguish them. Knight-Jones (1951) observed that "gregariousness .....is probably a general feature of the settlement behaviour of planktonic larvae, helping them to find suitable habitats, to maintain old breeding stocks and to form new ones". Working on the barnacle *Elminius modestus,* Knight-Jones & Stephenson (1950) showed that it too settled gregariously and Knight-Jones (1953), working on the barnacle *Balanus balanoides*, was able to add the facts that adult body fragments, when placed on various surfaces, made these surfaces more attractive for cypris settlement but that actual contact with an adult shell was necessary for settlement to take place. However, it was left to Crisp & Meadows (1962) to demonstrate the presence of a chemical 'settling factor' which could be shown to influence the settlement of barnacle cyprids.

Wilson (1968) carried out pioneering work on the settlement of *Sabellaria alveolata*, a sedentary polychaete which forms its tube by cementing together particles of sand. He found that 'slimed', i.e. bio-filmed, surfaces were not particularly conducive to settlement and that there was no preference for rough or smooth surfaces. Wilson (1970) went on to show that tube fragments crushed to sand attracted larvae to

settle preferentially and that physical factors have only a minor influence on settlement. Wilson was also able to show that the strongest stimulus for the larvae to settle was contact with the adult tube. and that the settlement factor which caused this stimulus was insoluble in water and unaffected by drying.

Nott (1973) produced a relevant paper on the settlement of *Spirorbis spirorbis* larvae including a detailed description of the settlement and subsequent tube formation. Nott & Parkes (1975) described calcium secretion in *Spirorbis spirorbis* and mentioned in passing that aggregates of calcium have been observed in the gut of *Pomatoceros triqueter*. Potswald (1978) described the settlement of *S. moerchi* which differs from that of *S. spirorbis* in the way in which the larvae attach to the substratum and in primary tube formation.

In addition to Knight-Jones (1951), many workers have noted the effect of biofilming on larval settlement, e.g. Zobell & Allen, 1935; Meadows & Williams, 1963; Wilson, 1968; Scheltema, 1974; Maki & Mitchell, 1985; Maki *et al.*, 1990. It is obvious that biofilming is an important factor in the settlement of the larvae of many, but not all, species of marine invertebrates.

In a major review of invertebrate larval settlement, Crisp (1974) brings together all the work to that date and provides a comprehensive overview of the subject. He provides many insights of his own on various topics and the work is also a valuable source of reference material. Scheltema *et al.* (1981), working on *Hydroides dianthus*, a species with many similarities to *P. triqueter*, found that larvae preferred to colonise surfaces inhabited by adults but that there was a wide variation in density of settlement.

Hadfield (pers. comm., 1992), working on the settlement of *Hydroides elegans*, found that the larvae settled highly preferentially on biofilmed surfaces and that this preferential settlement appeared to be closely related to the presence of a small, rod-shaped bacterium.

MATERIALS AND METHODS

Stones holding colonies of *Pomatoceros lamarckii* were collected from the Anglesey shore of the Menai Strait in the vicinity of the Telford suspension bridge. These stones were transferred to a tank of running seawater in the laboratory aquarium where they were kept until required.

Gametes were obtained by breaking pieces off the posterior ends of large *P. lamarckii* tubes until they were sufficiently reduced to allow the animal within to be pushed gently back by pressing on the operculum with a wire of suitable diameter. As the abdominal segments emerged from the posterior end of the tube mature gametes would be released from the gonoducts. These gametes were collected and placed into separate 25ml. glass beakers, each containing 5ml. fine filtered and ultra-violet irradiated seawater (FFSW). When sufficient ova had been obtained the sperm suspension was added to the beaker containing the ova, gently stirred and allowed to stand for ten minutes for fertilisation to take place. The water was then changed by pipetting off and replacing with fresh FFSW before covering the beaker containing the zygotes and placing it in a controlled environment (CE) cabinet at 18°C to allow the larvae to develop.

Pomatoceros species larvae become free-swimming in *circa* 16 hours after fertilisation. The fertilisation procedure was usually arranged so that the larvae would develop overnight. The day on which the larvae became

free-swimming was considered to be day one for experimental purposes. Small numbers of developing larvae were cultured in FFSW in 250ml. crystallising dishes and larger numbers in one litre beakers. All containers were covered to minimise evaporation and placed in a CE cabinet at 18°C with a 12:12 h. light:dark regime. Aeration was found to be unnecessary. Larvae were fed with a 1:1 mixture of two unicellular algae, *Tetraselmis chui* and *Rhinomonas reticulata*. This mixture was added to the water on day one at a concentration of 200 cells μl^{-1}. Culture water was changed every five days and fresh algae added.

Water changing was accomplished by using a simple tube with 90μm. plankton net glued to one end to act as a filter. Care was necessary to ensure that the larvae were not damaged during the filtration process. Ensuring that the filter was immersed in seawater while the water containing the larvae was gently poured through, prevented the larvae being crushed against the mesh. The larvae were then transferred to clean FFSW by inverting the filter and gently pouring FFSW through the plankton net.

Welsh slate was used as the substratum for the majority of the settlement experiments as, in the field, it is readily colonised by *Pomatoceros* species and other sessile invertebrates, e.g. barnacles. The slate is easily obtained locally, is relatively soft and so can be cut to any desired shape. In some early, exploratory experiments uncut fragments of slate, obtained from the Dinorwic quarry, were used but in the majority of experiments slates cut to 20 x 50 x 3.5mm. and 50 x 50 x 3.5mm. were used. In order to ensure that substrata were as near identical as possible, slates for definitive experiments were split from one block and cut to the above sizes by Inigo Jones Slateworks, Pen y Groes, Gwynedd. The slates

were finally rubbed down to a uniform finish (on the experimental surface only) on 320 grit wet and dry abrasive paper, cleaned ultrasonically and sterilised in a 2% Chloros (sodium hypochlorite) solution. Experiments for which slate was inappropriate as a substratum were normally carried out using glass microscope slides as the substratum.

Experiments to determine the effect of adult *Pomatoceros lamarckii* on settlement required uniform experimental surfaces with attached and established adults. These colonised surfaces were prepared in three ways:

(i) Juvenile *P. lamarckii* which had been allowed to settle on the base of the culture vessel had, after a few days growth, calcareous tubes which were sufficiently strong to be dislodged from the glass by pushing against the long axis of the tube. A hypodermic needle ground to a chisel point was found to be suitable for this purpose. Animals grown on a flat, glass surface naturally have a flat tube base which is ideal for re-attaching to another flat surface. The dislodged animals were pipetted up and replaced in the required position on the experimental surface (in FFSW containing food organisms) and weighted down with a small chip of slate. (Unless weighted the tube lifts from the substratum each time the branchial crowns are protruded to feed, so preventing attachment of new tube to the substratum.) After a few days, normal tube growth resulted in the animal becoming attached to the experimental surface. The chip of slate could then be removed and the animal grown on to the required size. This method was tedious and time consuming when mature adults were required.

(ii) When mature adults were required it was found to be more effective to prise suitable animals, complete with tubes, off stones, place them on the experimental surface and weight them down with chips of

slate. If the tube was shortened before removal from its original substratum, by carefully chipping away the posterior end until it was slightly too short for the animal, it was found that the animal was encouraged to make new tube, thus hastening the attachment process. Once the tube was attached the chip of slate could be removed. Using this method, animals suitable for experimental work could be available within fourteen days. The main disadvantages were the unevenness of the base of the tube, which made it difficult to hold the tube in the correct position on a flat surface, and the difficulty of placing a suitable chip of slate on the large, adult tube in such a way that it would resist the upward force generated by the emerging branchial crowns.

(iii) The disadvantages of method 2 were overcome by the use of a small spot of cyanoacrylate adhesive (Loctite Super Glue 3) to attach the tube of the animal to the substratum. This adhesive provides an adequate bond within seconds, is non-toxic to *Pomatoceros* species and allows the tube to be attached to the substratum in any desired position. A further advantage of gluing is that the animal does not have to be attached by its original tube base. If this base was a difficult shape it was sometimes preferable to attach the animal by the flatter side of its tube. When attachment was made in this way the animal rotated over a period of a few days, while producing new tube, until it lay once again dorsal surface downward with the keel of the tube uppermost. This method was found to be unsuitable for use with juveniles due to the tendency of the fluid adhesive to creep and cover the whole animal.

When animals had formed a natural bond to the experimental surface they were suspended in a tank of running seawater until required for use.

Microscopy

Light microscopy was carried out with a Leitz Dialux compound microscope and a Wild M3Z dissecting microscope. Photographs were taken with a Leitz Orthoplan photomicroscope using 35mm. film.

Specimens were prepared for scanning electron microscopy (SEM) by fixing in 2.5% glutaraldehyde in seawater (1.5h.), rinsing in distilled water (15 sec.) and then dehydrated through an ascending ethanol series (0.5h. each change) and finally through acetone (1h.) before critical point drying from liquid carbon dioxide in a Polaron E3000 critical point drying apparatus. Small specimens such as larvae are easily lost during preparation for SEM. To minimise such losses specimens were always processed in a small, acetone resistant, plastic tube (8mm diameter x 14mm long) with removable end caps. These end caps had their centre sections removed and 45µm. mesh plankton net fitted to allow free passage of liquids.

Following critical point drying specimens were attached to aluminium SEM stubs by means of double-sided 'Sellotape'. Finally, specimens were coated with gold using a Polaron E5000 sputtering system before viewing in a Cambridge Stereoscan S120 scanning electron microscope operated at 10Kv.

Experimental Procedures

1. Early attempts to obtain settlement

Small, clean pieces of rough slate and glass coverslips were introduced to culture vessels containing metatrochophore larvae of *Pomatoceros lamarckii* which were considered to be ready to settle (referred to

throughout this chapter as competent larvae). Some of these experimental substrata were raised from the base of the culture vessel in order to offer opportunities for larvae to settle on their undersides. Any larvae found to have settled were grown on until the resulting adult tubes were *circa* 20mm. in length. These adults were then used in experiments to determine the effect of adult animals on settlement.

2. Preparation and use of extracts

In attempts to discover a 'settlement substance' extracts were prepared from various parts of the body and tube of adult *Pomatoceros lamarckii*, from larvae and juveniles and from the algae used as food. These extracts were prepared by crushing the material in a variety of solvents using a mortar and pestle or, in the case of small amounts of soft tissue, in a Pierce Reacti-ware micro tissue grinder. During the crushing operation the temperature was kept at around 2°C by keeping the apparatus in crushed ice. The crushed mixture, when thoroughly homogenised, was centrifuged for five minutes at 9000g. in an Eppendorf 5412 centrifuge. The supernatant was then drawn off and either used immediately or stored in a freezer until required for use.

In use the extract was applied to dry, clean or biofilmed test surfaces with a cotton bud or small paintbrush. When extracts containing a volatile solvent were used they were allowed to air dry naturally. When water was used as a solvent the extract was dried in a cool airstream generated by a hair dryer. Test surfaces were given three coats of an extract, each coat being dried before the next was applied.

An experiment consisted of one or more treated surfaces (usually 20 x 50 x 3.5mm. slate), a control surface treated with the solvent alone and a

clean control surface. The surfaces were placed in a glass crystallising dish, containing FFSW and larvae judged to be competent were added. From time to time the surfaces were examined for settled larvae using a point light source set parallel to, and level with, the surface being examined. This low-angle light enabled settled larvae to be seen more easily by being thrown into relief.

3. Larval response to adults and whole adult extracts

In order to determine a) whether an attractant chemical was being released into the water column by adult *Pomatoceros lamarckii* and b) whether *P. lamarckii* extracts in the water column would attract settling larvae, a simple piece of apparatus, based on that used by Marsden (1991) to test the responses of the larvae of *Spirobranchus polycerus*, was constructed from plastic tubing (Fig. 1).

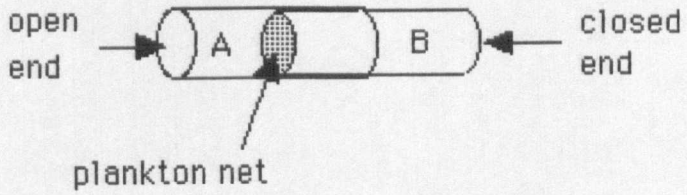


Fig. 1: Apparatus designed to show the presence of a water-borne attractant. Tube 'A' is open-ended and fits tightly over tube 'B' which is closed at the outer end and sealed by removable plankton net at the inner end.

Live adult *P. lamarckii* or *P. lamarckii* whole animal extract were placed in tube 'B' which was closed with a piece of plankton net held in place by tube 'A'. Control tubes, containing FFSW were also prepared and the pairs of tubes were placed in crystallising dishes containing competent

larvae. After 30 minutes the ends of the 'A' tubes were closed and any larvae within were counted.

4. Experiment to determine the duration of effectiveness of an attractant

Working on the hypothesis that an adsorbed chemical attractant on the tube was responsible for the attractiveness of the adult tube to competent larvae, small stones bearing adults with recent tube growth were taken from the field. The animals were removed from their tubes and all tubes except the one with most new growth were removed from each stone. One stone + tube was placed straight into a crystallising dish containing 100 competent larvae and this dish was placed into the CE cabinet on a 12:12h. light:dark regime. The remaining stones + tubes were put into a tank of running seawater. One stone + tube was removed from the seawater tank at 2, 4, 22, 24, 26, 28, 46, and 50 hours from the start of the experiment and placed into a crystallising dish with competent larvae as previously described. Commencing at hour 22, existing tubes were examined for settled larvae each time a new tube was introduced. Following the introduction of the final tube at 50 hours further examinations for settled larvae were made at 52 hours and 120 hours.

5. Exhalent current experiments

20 x 50 x 3.5mm. slates with attached adults were arranged alongside similar biofilmed slates without adults so that the exhalent stream from the adults passed over the biofilmed slates (Fig. 2). These pairs of slates were then put into the CE cabinet for seven days in order to allow the exhalent stream to condition an area of the slates.

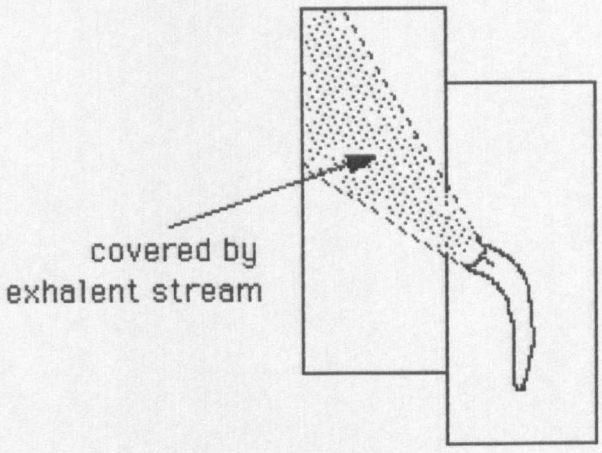


Fig. 2: Test slate arranged so that maximum coverage is attained by the exhalent stream.

The area covered by the exhalent stream could be determined from the faecal deposits and scale drawings were made of this area before the pairs of slates were introduced to crystallising dishes containing competent larvae. The positions of larvae which had settled were marked on the scale drawings and examined to determine if a settlement relationship existed for the area covered by the exhalent stream.

6. Larval conditioning experiments

50 x 50 x 3.5mm. slates were modified to hold larvae in a restricted area by milling a 12mm., flat-bottomed central pit 0.5mm. in depth (Fig. 3.(i)) Identical pits were later milled into other 50 x 50 x 3.5mm. slates 10mm. in from each corner (Fig. 3 (ii)). Horizontal and vertical lines were lightly engraved into the surface at 10mm. intervals in order to facilitate precise plotting of the positions of settled larvae. All prepared slates were biofilmed pit face downward in running seawater before use. A droplet of water containing *circa* 100 competent larvae was placed into

each of two central pits. A droplet of water which did not contain larvae was placed

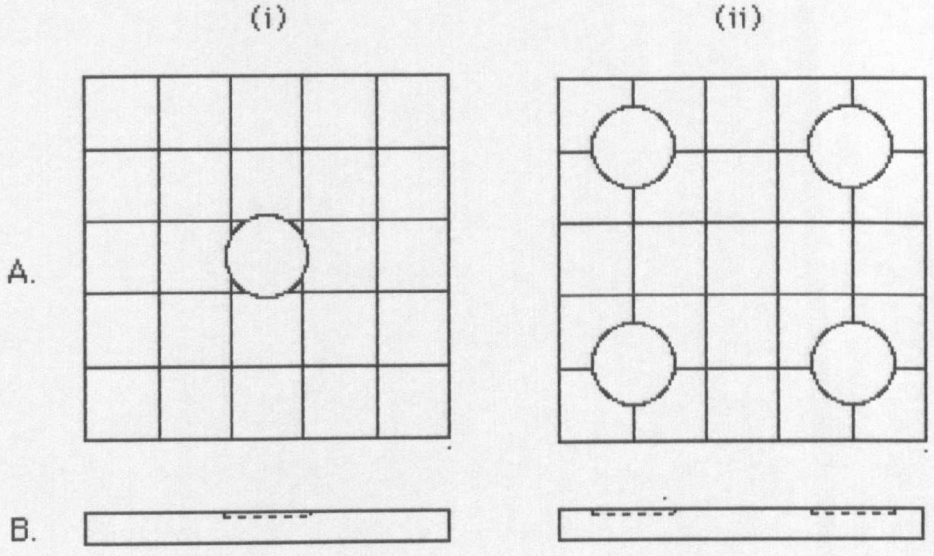


Fig. 3: Plan view (A) and side view (B) of slates prepared with 12mm. x 0.5mm. pits and engraved lines at 10mm. spacings.

into the pit of a third (control) slate. The slates were placed in covered petri dishes in the CE cabinet for four hours. At the end of this period the slates were gently flooded with FFSW to remove all larvae from the pits. Any settled larvae were removed and their positions marked on a scale drawing. The slates were then placed in crystallising dishes, each containing 200 competent larvae, and returned to the CE cabinet. Slates were examined and settled larvae plotted on scale drawings at 19, 22 & 44 hours.

The experiment was run twice in this form (pit experiments 1 & 2), the second experiment using the same set of slates which had been cleaned by vigorous scrubbing.

The experiment was then modified to include two controls, one biofilmed and one clean and was run three times in this form (pit experiments 3-5). All procedures other than the addition of an extra control were carried out as previously described.

The experiment was then again modified by the introduction of slates with four pits (Fig. 3 (ii)). This modification enabled each slate to carry two replicates, each consisting of one conditioned pit and one control pit. Four slates were used, three biofilmed and one clean control. Competent larvae were added to two pits on each slate including the clean control, a different pattern of larval:control pits being used on each slate. The same procedure as for the previous pit experiments was then followed. The experiment was run four times in this form (pit experiments 6-9). Experiment 7 was a repeat of experiment 6 using both the same slates, which had been lightly scrubbed in freshwater to remove larvae, and the same batch of larvae. Experiment 9 was a repeat of experiment 8 using both the same slates and the same batch of larvae. Settled larvae from experiment 8 were carefully removed with a dissecting needle and the slates were then gently rinsed in freshwater to remove any debris. These repeats were made in order to ascertain whether there was any correlation between the places where larvae settled in the first of each pair of experiments and where they settled in the repeat experiment.

In an attempt to show physical evidence of mucous deposits/trails left by larvae, droplets of water containing competent larvae were put into clean microscope cavity slides and left for five hours in the CE cabinet. At the end of this time the larvae were removed and the slides were gently rinsed in distilled water. Sets of such slides were stained with Alcian Blue

and Toluidine Blue for mucus and Bromphenol Blue for protein and examined under the compound microscope.

7. Biofilming experiments

A) Sterilisation of adult tubes

Four tubes (attached to 20 x 50 x 3.5mm. slates) from which the adults had been removed were stored for several months in darkness in FFSW. Two of these tubes were then sterilised by scrubbing vigorously in 100% methanol, scrubbing in running tap water and boiling for five minutes. They were then left for one hour to dry and then all the slates were put into a crystallising dish containing 800 trochophore larvae, FFSW and food in the form of a 1:1 mix of *Tetraselmis chui* and *Rhinomonas reticulata.* The crystallising dish was then placed in the CE cabinet on a 12:12 h. light:dark regime for 11 days before being examined for settled larvae.

B) Early slate biofilming experiment

This experiment used slates which had been biofilmed for differing periods. Two 50 x 50 x 3.5mm. slates were used, each with a central 12mm. pit (Fig. 3 (i)) which had been biofilmed, experimental surface downward, in the CE cabinet in the presence of adults for 44 days. Two identical slates which had been biofilmed, experimental surface downward, for 15 days in running seawater with no adults were also used. These four slates were each placed in separate crystallising dishes, each dish containing 200 competent larvae and food algae and placed in the CE cabinet in diffused light under the 12:12 h. light:dark regime. Slates were examined for settlers at 2 hours and 19 hours.

C) Definitive slate biofilming experiments

50 x 50 x 3.5mm. slates with an engraved grid and peripheral 12mm. pits (Fig. 3 (ii)) were used in the majority of biofilming experiments. Five of these slates were later cut into quarters, each containing a pit. All slates were biofilmed pit (experimental) face downward to reduce algal growth and the build-up of detritus on the experimental surface, but supported on small slate batons so that water could circulate freely. Sets of slates were biofilmed in four different environments:

- Running seawater.
- Running seawater containing adult *Pomatoceros lamarckii* on stones obtained from the field.
- FFSW containing the food algae *Tetraselmis chui* and *Rhinomonas reticulata* at a rate of 50 cells μl^{-1}.
- FFSW containing the food algae *Tetraselmis chui* and *Rhinomonas reticulata* at a rate of 200 cells μl^{-1} and adult *P. lamarckii* on stones obtained from the field.

A lower rate of food algae was used in the environment which contained FFSW and food algae only as these algae tended to proliferate in the absence of grazing by adult *P. lamarckii* .

The arrangement used to biofilm slates in running seawater is shown in Fig. 4. Slates biofilmed in running seawater only were placed in a 150mm. diameter crystallising dish supported in a 560 x 355 x 200mm. plastic tank. The slates to be biofilmed with adults were placed on the base of the plastic

tank and were surrounded by healthy adult *Pomatoceros lamarckii* growing on small stones collected from the Menai Strait.

Separate glass containers were used to biofilm the slates with and without the presence of adults in FFSW. Slates without adults were biofilmed in a 1 litre crystallising dish containing 900ml. of FFSW and slates with adults were kept in a 2 litre crystallising dish containing 1200ml. of FFSW. These containers were kept in the CE cabinet at 17°C with a 12:12 h. light:dark regime.

Those slates which were biofilmed in the presence of adult *P. lamarckii* were surrounded by small stones, collected from the Menai Strait, upon which healthy adults were growing.

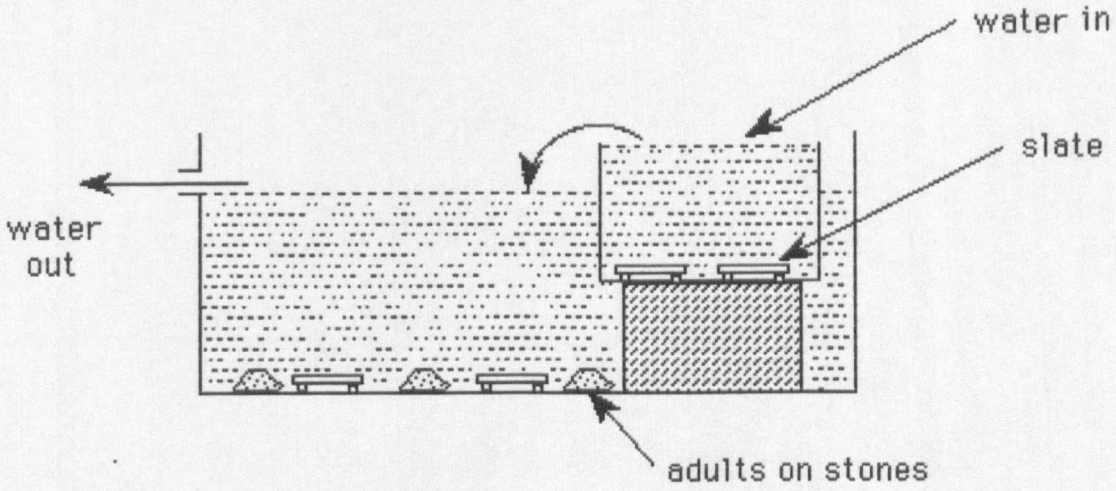


Fig. 4: The arrangement for biofilming slates in running seawater, with and without adults.

All surfaces were biofilmed for periods ranging from seven to nineteen days. It was not possible to run every experiment with slates which had been biofilmed for the same period of time as experiments had to be run when the larvae were judged to be competent and the time taken

to reach competence could vary from nine to >15 days. However, all slates used in any one experiment were biofilmed for the same length of time.

Immediately prior to an experiment slates from each of the biofilming environments were carefully examined for 'wild' settlers which might have settled during the biofilming period. The positions of any such settlers were marked on scale drawings and the settlers then carefully removed with a dissecting needle. The scale drawings were used to record the positions of settlers during the progress of the experiments. Initially the replicates were examined for settlers at 1, 2, 3, 4, 24 and 48 hours from the commencement of an experiment. Owing to the length of time required to examine each replicate, this examination period was later changed to 1.5, 3, 4.5, 24 and 48 hours in order to reduce the disturbance to the larvae.

Three sets of experiments were carried out:

(i) Substratum acceptability experiments

These experiments were designed to show the relative acceptability for settlement of each substratum.

Single 50 x 50 x 3.5mm. slates from each of the biofilming environments, plus a clean control slate, were placed into separate crystallising dishes containing 100ml. FFSW and food algae (*Tetraselmis chui* and *Rhinomonas reticulata)* at 200 cells μl^{-1}. 200 competent *Pomatoceros lamarckii* larvae were added and then the dishes were placed in the CE cabinet at 17.5°C with a 12:12 h. light:dark regime. This experiment was repeated three times with slates biofilmed for 7, 13 and 18 days respectively.

(ii) Substratum choice experiments

These experiments were designed to show which biofilmed substrata were preferred when a free choice between these substrata was available. Single 50 x 50 x 3.5mm. slates from each of the biofilming environments, plus a clean control slate, were placed into a single, large (190mm. diameter) crystallising dish containing 500ml. of FFSW and 100ml. of food algae. The slates were arranged as shown in Fig. 5 with the control slate centrally located. 300 competent *Pomatoceros lamarckii* larvae were added centrally and then the dish was placed in the CE cabinet at 17.5°C with a 12:12 h. light:dark regime. At each inspection the crystallising dish was rotated 90° in a clockwise direction (as viewed from above) and the individual slates were also rotated 90° clockwise. This procedure was carried out in order to eliminate any possible bias in the direction of irradiance within the CE cabinet. This experiment was repeated three times with slates biofilmed for 16, 13 and 10 days respectively. The results were statistically analysed by Chi^2 (goodness of fit to a uniform distribution) analysis.

(iii) Substratum choice/small slates

One possible criticism which could be levelled at the substratum choice experiments is that all the larvae may not have the opportunity to visit all the test substrata due to the size of crystallising dish required to accommodate all the slates. Observations of the larvae during these experiments and on other occasions showed them to range freely and do not lend support to this criticism. Having a mean swimming speed of

1.03mm. sec^{-1} (Chapter 3) a larva could cross the crystallising dish in *circa* three minutes and should therefore be able to explore all surfaces during the time span of an experiment. A more practical consideration was the difficulty of examining the slates *in situ* , for settled larvae, in such a large container during the course of the experiment. Therefore a set of 50 x 50 x 3.5mm. slates was cut into quarters and biofilmed as detailed above. This procedure allowing an experiment to be carried out using four small replicates within which the larvae could easily explore all surfaces and which were relatively easy to examine for settlers.

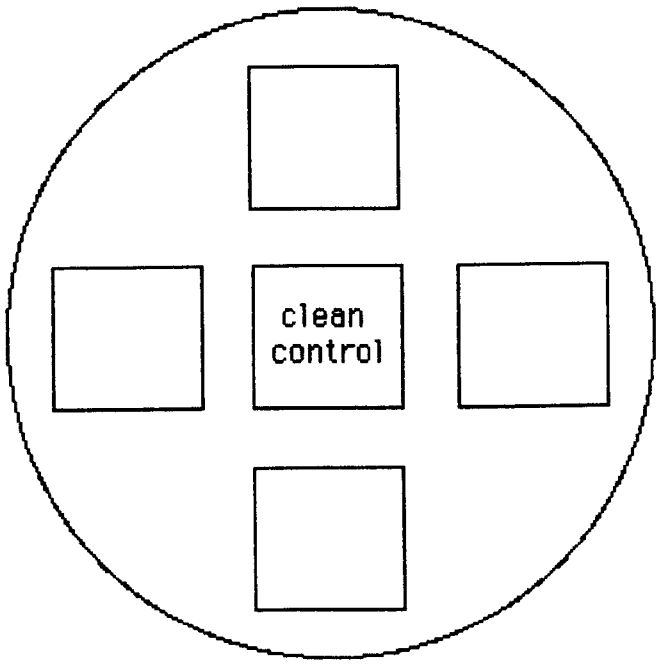


Fig. 5: The distribution of test substrata in choice experiments.

The cut slates were biofilmed for 19 days and then placed into four 90mm. diameter crystallising dishes containing 100ml. of FFSW and 25ml. of food algae and arranged as in Fig. 5. The four treatments were arranged in different positions in each replicate but the control was always

placed in the central position. 200 competent *Pomatoceros lamarckii* larvae per dish were added and the replicates placed in the CE cabinet at 17°C with a 12:12 h. light:dark regime. This latter experiment was only run on a single occasion. The results were statistically analysed by Chi^2 (goodness of fit to a uniform distribution) analysis.

D. Glass biofilming experiments

Sets of clean 25 x 75 x 1mm. standard glass microscope slides were sterilised in a 2% Chloros solution and then biofilmed as described above for the slate surfaces. After biofilming for 14 days one set of the slides was stained for the presence of acid mucopolysaccharides using equal volumes of 4% glutaraldehyde in phosphate buffer, 0.1M phosphate buffer (pH 7.4) and Ruthenium Red at 1500 ppm. Following staining in this solution for one hour the slides were given three rinses in phosphate buffer. The stained slides were examined under a compound microscope for the presence of bacteria and six random photographs per slide were taken of the bacterial coverage.

After biofilming for 15 days a second set of the slides was stained to show bacterial cell walls. The slides were fixed for one hour in Bouins fixative, rinsed in distilled water, stained in Crystal Violet for one minute and then rinsed in distilled water to remove any surplus stain. These slides were again examined for the presence of bacteria and photographs taken as before. Permanent mounts of the slides were then made and examined under the compound microscope, using an oil immersion lens, to determine the types of bacteria present on each. Bacterial counts were made in selected 10mm. squares of each photograph. Three counts were made in

densely populated areas of each photograph and three in sparsely populated areas. The mean count per $10\mu m^2$ was then calculated.

RESULTS

1. Early attempts to obtain settlement

Settlement on clean experimental surfaces was very low. From four experiments using a total of 1100 larvae only 28 larvae (2.6%) settled. The majority (sixteen) of these larvae settled on the bases of the culture vessels. A further eleven larvae settled on pieces of slate, six of these larvae being found on the sides and underside of the slate, and one larva settled on a glass coverslip. When metatrochophore larvae were introduced to substrata bearing *Pomatoceros lamarckii* adults the results were strikingly different (Table 1).

Table 1: Numbers of settled *Pomatoceros lamarckii* larvae in relation to *P. lamarckii* adults attached to slate and glass coverslips. sub. = substratum.

Adult 1 on slate sub.		Adult 2 on slate sub.		Adult 3 on glass sub.		Adult 4 on glass sub.		Base of container
Adult tube	Slate sub.	Adult tube	Slate sub.	Adult tube	Glass sub.	Adult tube	Glass sub.	Glass
208	371	50	3	98	14	51	1	5

The settlers on the slate substratum of adult 1 were found on both upper and lower surfaces of the slate, 259 being found on the upper and 112 on the lower surface. *Circa* 1100 larvae were introduced to the container but direct comparisons between numbers of settlers on surfaces cannot be made as, apart from the coverslips, all surfaces were of different areas, e.g. adult 2 virtually covered the slate to which it was attached, leaving little space for settling larvae. The most obvious observation is that adult tubes of *Pomatoceros lamarckii* were very attractive to settling *P. lamarckii* larvae and that the substrata to which the adults were attached also proved attractive. In most cases the adult tubes attracted the most settlers but the slate substratum of adult 1 proved more attractive than the tube of the animal.

These observations were repeated on many occasions, usually as part of other experiments using a more controlled experimental design. The relationship between settlement on tube and substratum always proved to be variable, with the adult tube proving to be more attractive than the surrounding substratum on some occasions and *vice versa* on others.

Having established that adult tubes and the substrata immediately around them were highly attractive to settlers it was then possible to observe settlement behaviour in some detail.

2. Settlement Behaviour

Metatrochophore *P. lamarckii* larvae were usually ready to settle between nine and fourteen days from becoming free-swimming but were able to delay settlement for quite a considerable time in the absence of a

suitable substratum/stimulus. Given favourable conditions larvae settled readily while still completely mobile and before the prototrochial cilia were lost. It would appear that it is only when conditions are unsuitable for settlement that larvae metamorphose before tube formation as described by Segrove (1941) (see Chapter 3).

When competent, larvae often swam in an undulating path which, in shallow water, took them alternately close to the substratum and to the water surface. Occasionally they swam in spirals with little rotation about the long axis. Rotation about the long axis does occur when larvae lose a section of the prototrochial cilia, one of the early signs of metamorphosis. In the present study strong negative phototaxis was always found to be a precursor of settlement.

There appeared to be three phases of searching behaviour. The terminology used by Crisp (1974) to describe larval searching patterns, 'broad exploration', 'close exploration' and 'inspection', is appropriate to the present study. During broad exploration larva would swim rapidly over a wide area, pausing occasionally in a vertical, head down position with the apical cilia in contact with the substratum. If an 'attractive' surface was encountered the close exploration phase ensued. The animal stopped swimming and began gliding rapidly over the surface, using the neurotroch cilia as the main means of propulsion. The ventral area of the head was maintained in close contact with the substratum. Occasionally the animal would adopt the head-downward position with the apical cilia in contact with the substratum. If the surface proved to be unsatisfactory the animal would glide or swim rapidly away. Movement during the close exploration phase could be quite wide ranging but on receiving a suitable positive stimulus the inspection phase commenced and the animal

concentrated its search into an area of one to two millimetres square. This area would be repeatedly traversed in all directions for several minutes before fixation took place.

During the phases of searching described above, some larvae could be seen to be trailing a strand of mucus from the posterior end, presumably emanating from the anal vesicle. Occasionally a larva would be seen to tail-fix to the substratum by this mucous strand but such attachments were always temporary. No evidence was found to support Moat's (1985) hypothesis that the anal vesicle and mucous glands of the anal area play a major part in permanent attachment.

Finally, when a site was chosen, the larva usually turned on the spot two or three times before orientating its body in the desired direction with the ventral surface in contact with the substratum. Just prior to fixation the anterior part of the body was raised sharply at right-angles to the substratum. The animal settled back into position and was immediately firmly attached, as judged by the fact that it could not be dislodged by a water jet from a Pasteur pipette. Attachment was by a large pad of mucus in the area of the ventral shield epithelium (Fig. 6). It was sometimes noted that a tail attachment was made just before the body was raised at right angles. It is quite possible that this temporary attachment is necessary to hold the larva at the chosen settlement site while the permanent attachment is made.

Larvae settling on adult tubes usually showed a marked preference for the anterior end of the tube, close to the collar and the first settlers almost invariably chose the depression between the keel and the apex of the tube as a settlement site. The contour between the base of the tube and the substratum was also regularly colonised by early settlers. Once a larva

had settled it was not unusual for another to settle alongside. Despite the apparent preference for a concavity, those larvae which settled on the slate did so mainly on the flat surfaces.

As the adult tube was clearly attractive to settlers in the laboratory, adults were collected from the field and examined in order to ascertain whether this preferential larval settlement also occurred in the wild. Due to the way in which many tubes are intertwined in areas of dense settlement it was not always possible to determine the point of origin of many animals. Therefore observations were limited to those animals in which the area of origin could clearly be seen. It was obvious that many of the juveniles had originally settled on, or very close to, adult tubes (Fig. 7). These and other field observations are more fully dealt with in Chapter 6.

3. Early Tube Formation

As previously noted, larvae will settle readily while still actively free-swimming if presented with an 'attractive' substratum. Once settled the prototroch rapidly disappeared, the head area became more clearly defined and the collar unfolded to enclose the anterior end of the mucous tube.

Having attached itself to the substratum, a larva would commence to rotate about its long axis, making one complete 360° rotation in one direction followed by another in the opposite direction. Simultaneously it moved rhythmically backwards and forwards along its long axis, moving a total distance of approximately 200 μm. These movements continued vigorously for some time, the animal always returning to a ventral surface down orientation. Careful observation at this stage showed the animal to be rotating within a transparent mucous tube which could be detected by

the presence of small reflective areas, which remained stationary in relation to the movements of the body. The presence of this mucous tube was confirmed by scanning electron microscopy (Fig. 8).

Within 15 to 30 minutes a small, semi-opaque tube around 75μm. long was formed around the thoracic segments. The animal then moved and rotated much less frequently and rested (now dorsal surface downwards) for long periods. The abdominal area, though coated in mucus was free and was often raised above the surface (Fig. 6). This initial tube was round and did not have the characteristic keel (Fig. 9). The tube was not completely calcified when the base was in contact with the substratum, the basal area being composed of mucus alone. As this mucus is transparent it appears that the tube is incomplete (Faouzi, 1931; Thomas, 1940). If the anterior end of the tube was not in contact with the substratum, as was the case when larvae settled on top of previously settled larvae, the tube remained circular externally and the whole of the circumference became calcified. This observation does not accord with that of Faouzi (1931) but confirms that of Thomas (1940). Observations of several animals have shown that the time from investigation of the final settlement area to formation of the initial semi-opaque tube ranged from 25 minutes to one hour.

Early tube growth was rapid, the length of semi-opaque tube increasing to *circa* 300 μm. in three to four hours. Initially calcium carbonate production was low and the tube remained semi-opaque, with the body of the animal visible within, for several days. Externally the tube was circular for four to five days until the collar was sufficiently developed to allow keel formation. As the keel started to appear the tube gradually assumed a more triangular external section while the interior remained round. As might be expected, tube growth is rapid while the

Figure 6.

Scanning electron micrograph of a *Pomatoceros lamarckii* juvenile 1 hour after settlement. A large mucus pad (arrow) anchors the animal to the substratum. The abdominal segments are concealed by the primary mucus tube, over the anterior end of which is the collar (c). the animal has settled in the angle between the keel (k) and the sidewall of the tube of a conspecific adult. Scale bar...50 μm.

Figure 7.

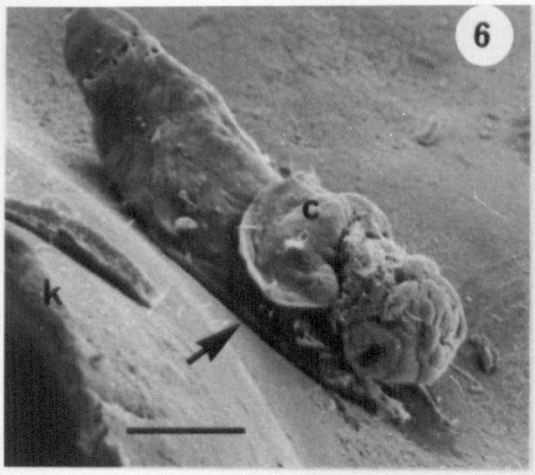
Light micrograph showing two juvenile *Pomatoceros lamarckii* which have settled in the angle between the base and sidewall of the tube of a conspecific adult (on a stone from the Menai Strait) and have then commenced to grow away from the adult tube.
Scale bar...1.2 mm.

Figure 8.

Scanning electron micrograph of a *Pomatoceros lamarckii* juvenile, processed 6 minutes after settlement. A thin primary tube, firmly anchored by a mucus sheet (ms) at the posterior end, covers the body segments. The remains of the prototroch (p) can be seen. Compare with Fig. 6 where the posterior end of the primary tube is not anchored. Scale bar...50 μm.

Figure 9.

Light micrograph of a juvenile *Pomatoceros lamarckii* tube. The anterior portion is lightly calcified and a long primary mucous tube can just be discerned. The operculum (o) also has a calcified band. Scale bar...200 μm.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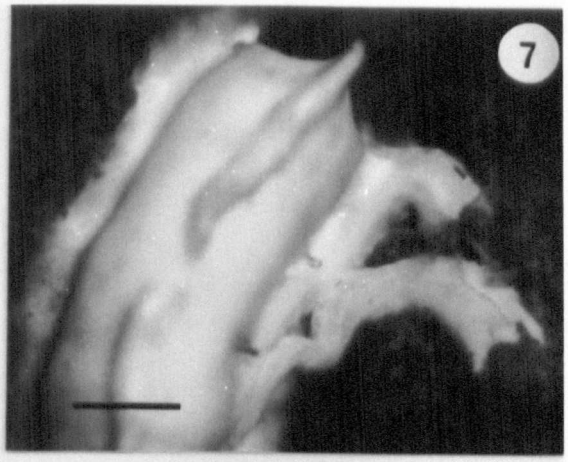
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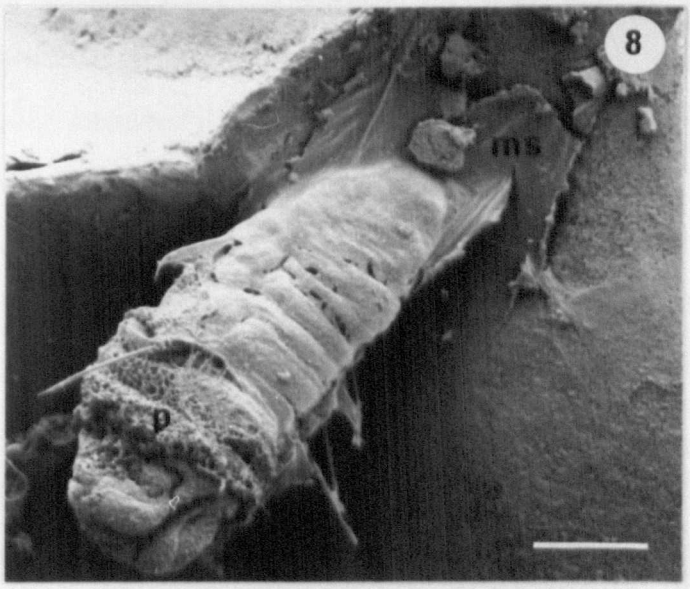
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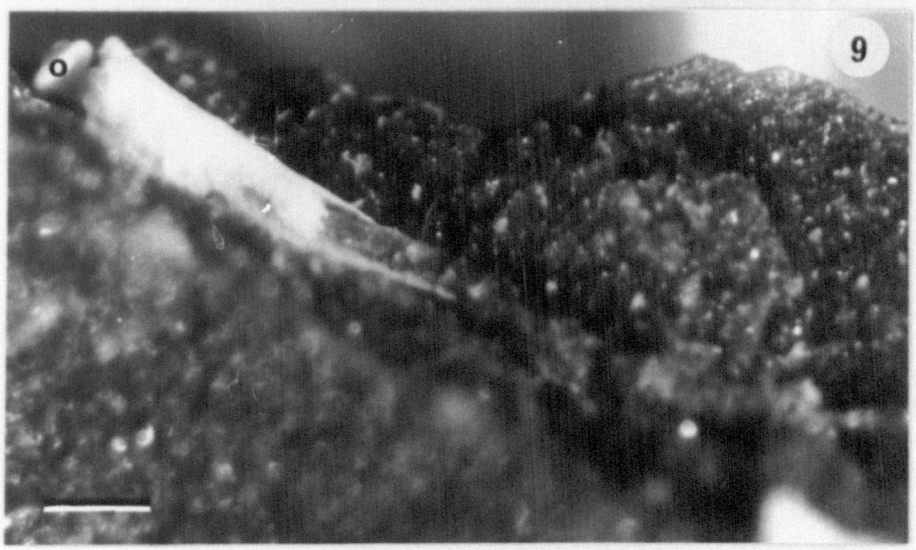
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o

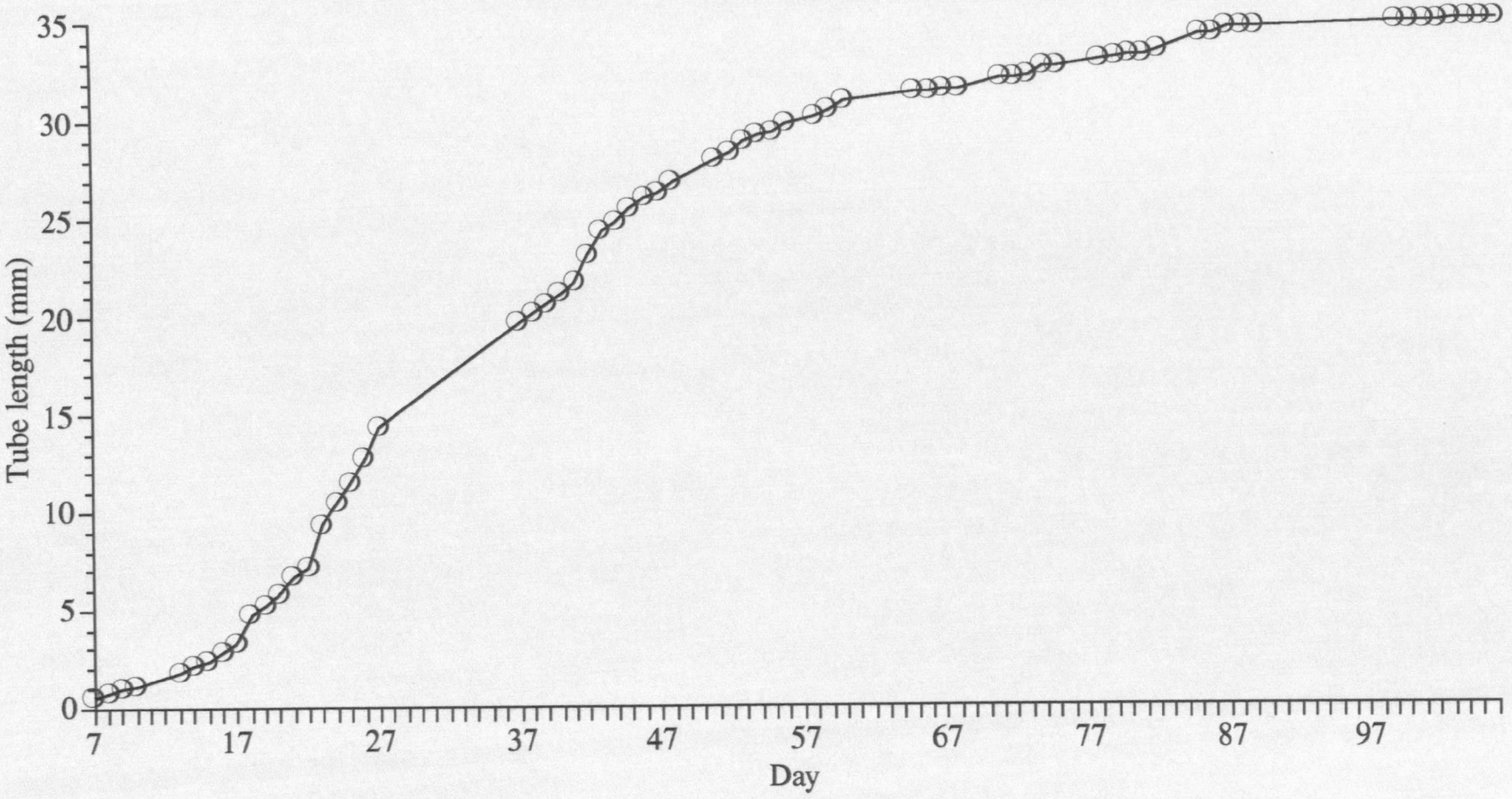
animal is also growing rapidly. At around 60 days tube growth slows and further tube growth is sporadic with long periods when no growth occurs (Graph 1).

It was noticed that, when the semi-opaque tube was *circa* 125-175 µm. in length, the posterior end of the animal was often still pointed, suggesting that the anal vesicle still contained mucus. At this early stage the posterior end of the tube often remained open and the animal could be teased out backwards with the eyelash tool. Vigorous attempts would be made to regain a position in which the collar was over the mouth of the tube. These attempts were usually successful, provided that the animal had not been completely removed from the tube. Animals which were completely removed from their tubes could, at this stage, occasionally construct a new tube and re-attach to the substratum. This ability is in marked contrast to mature adults which, under normal circumstances, cannot make a new tube or re-attach once evicted from their tubes (Thomas, 1940).

Thomas (1940) noted that a series of cavities exist internally in the sides of the tube at the base. She suggests that these cavities are probably formed to economise on material. If larvae are induced to settle on glass coverslips these cavities can easily be observed from the underside (Fig.10).

4. The use of extracts to attempt to identify a 'settlement substance'

A variety of parts of adult and juvenile animals, larvae and single celled algae was used in a variety of solvents. The results of the experiments carried out are summarised in Table 2.



Graph 1: Tube growth of a *Pomatoceros lamarckii* juvenile from day 7 from settlement to day 106

The only extracts which appeared to have any effect on settlement were those made from metatrochophore larvae and unicellular food algae (*Tetraselmis chui and Rhinomonas reticulata*). All other extracts either attracted no settlers at all or so few that they could be discounted as random settlers.

Table 2: A summary of the experimental attempts to find a crude settlement substance using *Pomatoceros lamarckii* tissue extracts in a variety of solvents.

Solvent	Part of organism used	Total number of replicates	Result
Distilled water	Whole animal including tube	4	No significant settlement on treated surfaces (NSS)
Distilled water	Tube (new)	3	NSS
FFSW	Whole animal including tube	2	NSS
FFSW	Whole animal	9	NSS
FFSW	Collar	4	NSS
FFSW	Tube (Old)	3	NSS
FFSW	Tube (newly formed)	7	NSS
FFSW	Tube (whole)	4	NSS
FFSW	Newly settled juveniles	1	NSS

Table 2 cont.

Solvent	Part of organism used	Total number of replicates	Result
Di-methyl sulphoxide (DMSO)	Tube (whole)	5	NSS
DMSO	Whole animal	4	NSS
DMSO	Thoracic area	1	NSS
Di-ethyl ether	Whole animal including tube	1	NSS
Di-ethyl ether	Whole animal	1	NSS
1:1 chloroform/ methanol	Whole tube	1	NSS
1:1 chloroform/ methanol	Thoracic area	1	NSS
Distilled water	Unicellular (food) algae	3	No definite preference for treated surfaces
FFSW	Metatrochophore larvae	21	Some settlement on nine replicates
FFSW	Unicellular (food) algae	12	Settlement on treated surfaces in all except three experiments

When settlement on an extract-treated surface was very poor or nil this result could, of course, be due to the effect of a poor batch of larvae or larvae which were not competent. In order to test for this possibility slates with attached adults were sometimes introduced at the termination of an experiment and later checked for settled larvae. Table 3 shows the result of such a test. The final column shows the total number of larvae which settled on the adult tube and on the substratum surrounding it. Obviously, in this case, the larvae were competent given a suitable stimulus. Should the larvae not settle on an adult or the substratum to which it was attached, it could reasonably be assumed that the result was due to a poor batch of larvae.

Table 3: The effect of introducing an attached adult at the termination of an experiment with zero settlement. This particular experiment had an initial input of 178 larvae.

	Treated surface	Biofilmed control	Clean control	Introduced adult tube and substratum
No. of settlers	0	0	0	88

In addition to the use of solvents to attempt to extract a settlement substance, the following techniques were also employed:

- Swabs were taken from beneath the collars of adult worms and smeared on test surfaces (15 replicates).
- Swabs were taken from the outer surfaces of newly formed tube and smeared on test surfaces.
- Scrapings from the surface of tubes were spread on test surfaces.
- Adult worms were removed from their tubes, placed on test surfaces and rolled about in a marked area in order to spread any mucus or other secretions.
- Adult worms were removed from their tubes, kept in a small volume of FFSW (11 adults in 1.5ml. FFSW for two hours) and this FFSW was then painted onto test surfaces and allowed to dry.

None of these methods resulted in any settlement on the test surfaces. However it was noted that in a number of experiments where biofilmed surfaces had been used the larvae settled readily on such surfaces.

These results suggested that either a 'settlement substance' did not exist, or that it was not soluble in a range of solvents, or that it was adversely affected by the methods used in its isolation. Further experiments were then designed to attempt to show the presence of a 'settlement substance'.

5. Attempts to show the presence of a 'settlement substance'

A. Tube plugging experiments

Adults which were actively making new tube were selected and the tubes of half the selected animals were plugged with Plasticine modelling clay to prevent the animal emerging during the experiment. The tubes

were then introduced to containers holding competent larvae. Both plugged and unplugged tubes were settled indiscriminately which suggested that the adult had no direct effect on settlement and was not emanating a water borne chemical attractant.

B. Newly-vacated versus 'old' empty tubes

Old tubes attached to 20 x 50 x 3.5mm. slates, which had been stored for some months in the dark in FFSW were compared, for their attractiveness to settling larvae, with tubes from which the adults had just been removed. The results of these experiments were rather variable. In general, a newly-vacated tube would be settled in preference to an old tube, but occasionally an old tube would be settled by large numbers of larvae.

C. Introduction of adults into old tubes

Adults were removed from their tubes and introduced to old tubes attached to 20 x 50 x 3.5mm. slates, which had been kept for some months in the dark in FFSW water. These tubes and an equal number of similar old empty tubes were kept overnight in FFSW containing food algae then introduced to containers holding competent larvae. The results showed that larvae settled on the old tubes containing adults in significantly greater numbers than those tubes without adults.

D. Larval response to adults and whole adult extracts

The larvae were found to be equally distributed between the tubes containing the test materials and the control tubes thus demonstrating that a

water-borne attractant was not being produced by the adults and also that adult extract in solution had no attractive effect.

E. Experiment to determine the duration of effectiveness of an attractant

This experiment was carried out on two separate occasions, the initial experiment being run for only four hours when a good response suggested that the experiment should be run for a longer period. Good settlement was obtained on all tubes up to the full 50 hours, suggesting that the attractant was long lasting in normal conditions. It was noted on this occasion, as on other occasions, that no settlers were to be found on the insides of the tubes. This lack of settlement could have been due to an absence of attractant, or to a larval preference for the external surface, perhaps occasioned by a greater concentration of attractant. Therefore the following experiment was designed and run.

F. Settlement on the inner surface of *Pomatoceros lamarckii* tubes

Newly formed tubes, from which the animals had been removed, were cut longitudinally and then one half of the cut tube was removed to expose the inner surface of the remaining half. This remaining half was then introduced to a crystallising dish containing competent larvae.

This experiment was only carried out once, using two replicates and there was a very low rate of settlement (2% & 5.75%). However, the results showed that larvae will settle on the inner surface of the tube if it is accessible. The majority of larvae which settled on the inner surface settled on the sidewall in close proximity to the base of the tube.

G. Exhalent stream experiments

These experiments were repeated eight times. The distribution of the settled larvae showed no clear relationship to the areas of slate which had been exposed to the exhalent stream. It was, however, clear that surfaces which had been biofilmed in the presence of adult *Pomatoceros lamarckii* were more attractive to settlers than those biofilmed without the presence of adults.

H. Larval Conditioning Experiments

The results of the first experiment were encouraging (Table 4).

Table 4: Settlement of *Pomatoceros lamarckii* larvae on slates with larval conditioned pits. First experiment.

Time (h)	Replicate 1.		Replicate 2.		Biofilmed control	
	Pit	Slate	Pit	Slate	Pit	Slate
19	6	0	13	3	0	0
44	7	0	13	11	1	6

At 19 hours the preference of the settling larvae was clearly for the larval conditioned pits, with the angle between edges and bases of the pits appearing to be particularly attractive, but at 44 hours there were also settlers on the slate of replicate 2 and on the control suggesting that, although the primary attraction was to the conditioned pits, the effect of biofilming had a part to play in the result. This hypothesis was tested by

vigorously scrubbing the slates before carrying out the second, otherwise identical, experiment.

The result of this second experiment was that there were only two settlers from a total of 600 larvae, one settler in a treated pit and one on the control slate. This result appeared to confirm the hypothesis that the initial result was dependent on biofilming.

The findings of the first two experiments suggested that more experiments should be carried out using both biofilmed and clean controls. A poor settlement on the clean controls would support the role of biofilming in the settlement process. Accordingly three more experiments were run, as described in Materials and Methods, using an additional, clean control. The results of these experiments are summarised in Table 5.

Table 5: Settlement of *Pomatoceros lamarckii* larvae on slates with larval conditioned pits. Experiments 3 - 5.

Expt.	Replicate 1		Replicate 2		Biofilmed control		Clean control	
No.	Pit	Slate	Pit	Slate	Pit	Slate	Pit	Slate
3	6	4	56	4	1	29	0	0
4	18	9	9	12	10	8	0	1
5	26	19	69	8	22	47	0	0
Total	50	32	134	24	33	84	0	1

These results clearly show that biofilming has an important role in the settlement process. The clean control attracted only one settler from three experiments using, in all, 600 larvae whereas the biofilmed control

attracted a total of 117 settlers. What is not quite so clear is the effect of the conditioning larvae used in the pits of replicates 1 & 2. There is a difference between the number of settlers in the conditioned pits and the number of those in the biofilmed pits but this result has to be seen in the context of the fact that each replicate was in a different container. Given a choice between biofilmed and biofilmed + larval conditioned pits would the result be the same? To answer this question the experiment was re-designed using slates with four pits (Fig. 3 (ii)) allowing two replicates per slate and a choice between conditioned and biofilmed pits. This experiment was run four times with the results shown in Table 6.

It can be seen that there was very poor settlement in experiments 6 and 7, probably due to the batch of larvae not being competent, and thus no useful conclusions could be drawn from these experiments. In experiments 8 and 9 settlement was not high but some useful observations may be made. The first of these observations is that, with larvae free to choose between treated and untreated pits, there is no obvious preference for the larval conditioned surfaces, suggesting that biofilming and surface contour, rather than larval conditioning, are the factors influencing settlement in this series of experiments. Of these two factors it would appear that biofilming is of far greater significance than surface contour as there were no settlers on the clean controls.

No correlation was found between the positions of settlers in the first of each pair of experiments and positions of settlers in the repeat experiments.

Examination of cavity slides in which the cavities had been conditioned by competent larvae and then stained for mucus and protein, revealed that the larvae did leave deposits and trails on the substratum. These secretions

Table 6: Settlement of *Pomatoceros lamarckii* larvae on slates with larval conditioned pits. Experiments 6-9.
TP = treated pit. **C** = control. **Sl** = slate.

Expt. No.	Biofilmed slate 1					Biofilmed slate 2					Biofilmed slate 3					Non-Biofilmed control slate				
	TP	TP	C	C	Sl	TP	TP	C	C	Sl	TP	TP	C	C	Sl	TP	TP	C	C	Sl
6	1	0	4	0	5	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0	3	0	0	0	0	1	0	0	0	0	0
8	6	5	0	7	16	2	4	2	2	18	3	1	2	0	13	0	0	0	0	0
9	1	4	2	2	3	9	3	13	1	68	9	0	8	1	79	0	0	0	0	0
Totals	8	9	6	9	24	11	7	15	3	92	12	1	10	1	93	0	0	0	0	0

stained well with Alcian Blue and Toluidine Blue but not with Bromphenol Blue indicating that they consisted essentially of mucus and not protein.

6. Biofilming Experiments

A. Sterilisation of adult tubes

The number of settled larvae was extremely low: 3 larvae in replicate one and 34 larvae in replicate two out of a total of 800 larvae in each replicate. Therefore no sound conclusions may be drawn from this experiment. It is, however, worthy of note that no larvae settled on the tubes and slates which had been sterilised.

B. Early slate biofilming experiment

Examination at two hours showed very heavy settlement on the slates biofilmed in the presence of adults (Table 7). The settlement situation was little changed at 19 hours and the experiment was terminated at that point.

There was a dramatic difference between the number of settlers on the adult biofilmed surfaces and those biofilmed in running seawater only, also the percentage settlement on the adult biofilmed slates was remarkably high. The majority of those larvae which settled in the pits preferred the angle between the base and the side of the pit.

Table 7: The settlement of *Pomatoceros lamarckii* larvae on surfaces biofilmed in and without the presence of adults.
ABS = surface biofilmed in the presence of adults.
RSW = surface biofilmed in running seawater only.

Time	ABS		ABS		RSW		RSW	
(h)	Pit	Slate	Pit	Slate	Pit	Slate	Pit	Slate
2	18	128	47	125	0	0	0	0
19	19	146	47	131	5	6	3	2
% Settlement	82.5		89.0		5.5		2.5	

C. Definitive slate biofilming experiments

(i) Substratum acceptability experiments

Settlement was not high in any of these experiments, but slates which had been biofilmed in the presence of adults were clearly more attractive than those which had not (Table 8). There was little to choose between the attractiveness of surfaces biofilmed in running seawater and those in FFSW. The latter made up 51.4% of all the settlers and the former 44.9%. The only other slate to have any settlers was that biofilmed in running seawater which had 3.7% of the settlers.

(ii) Substratum Choice Experiments

Settlement was 49.1% in these experiments with, again, the slates biofilmed in FFSW + adults + algae receiving the greatest number of settlers (Table 9). Chi^2 analysis of the data showed that there was a significant difference in the number of settled larvae on the five surfaces

Table 8: The settlement of *Pomatoceros lamarckii* larvae on slates biofilmed with and without the presence of adults in FFSW and running seawater:
Substratum acceptability experiments, results at 48 hours.
FFSW = fine filtered seawater. **P1- P4** = pits 1-4 **Sl** = remaining slate surface.

Expt. No.	Slate biofilmed in FFSW + adults + algae					Slate biofilmed in Running seawater + adults					Slate biofilmed in Running seawater only					Slate biofilmed in FFSW + algae					Clean control slate				
	P1	P2	P3	P4	Sl	P1	P2	P3	P4	Sl	P1	P2	P3	P4	Sl	P1	P2	P3	P4	Sl	P1	P2	P3	P4	Sl
1	4	0	2	0	5	0	2	8	10	7	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
2	2	0	0	0	12	2	0	11	6	2	1	0	0	2	0	0	0	0	0	0	0	0	0	0	0
3	8	4	0	4	14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Totals	14	4	2	4	31	2	2	19	16	9	1	1	0	2	0	0	0	0	0	0	0	0	0	0	0

Table 9: The settlement of *Pomatoceros lamarckii* larvae on surfaces biofilmed with and without the presence of adults in FFSW and running seawater: substratum choice experiments, results at 48 hours.

FFSW = fine filtered seawater. **P1- P4** = pits 1- 4. **Sl** = remaining slate surface.

Expt. No.	Slate biofilmed in FFSW + adults +algae					Slate biofilmed in Running seawater + adults					Slate biofilmed in Running seawater only					Slate biofilmed in FFSW + algae					Clean control slate				
	P1	P2	P3	P4	Sl	P1	P2	P3	P4	Sl	P1	P2	P3	P4	Sl	P1	P2	P3	P4	Sl	P1	P2	P3	P4	Sl
1	0	0	1	1	6	6	13	4	12	31	3	2	4	9	5	0	0	0	0	3	0	0	0	0	1
2	5	8	17	31	145	0	0	0	0	0	0	0	0	1	3	0	0	0	0	0	0	0	0	0	0
3	17	13	7	2	84	1	0	0	0	0	3	0	0	0	4	0	0	0	0	0	0	0	0	0	1
Total	22	21	25	34	235	7	13	4	12	31	6	2	4	10	12	0	0	0	0	3	0	0	0	0	2

(probability < 0.05). Taken on the total figures for the three experiments the descending order of biofilm preference was still; FFSW + adults + algae ➡ running seawater + adults ➡ running seawater only ➡ FSSW + algae ➡ clean control, but in this series of experiments the differences between the numbers of settlers on the various biofilmed surfaces, expressed as percentages of the total number of settlers, were greater: 76.1% ➡ 15.1% ➡ 7.7% ➡ 0.7% ➡ 0.4%.

When setting up experiment 1, two 'wild' settlers were found in pit 3 of the slate biofilmed in running seawater only. None of the larvae which subsequently settled during the experiment chose the same sites as the 'wild' larvae.

When setting up experiment 2, the slate which had been biofilmed in running seawater only had fifteen 'wild' settlers and two *Spirorbis* species settlers in pit 1, two *Spirorbis* species settlers in pit 2 and two 'wild' settlers plus one *Spirorbis* species settler on the slate. Pits 1 & 2 attracted no settlers during the experiment and the few larvae which settled on the slate were not attracted to the positions which the 'wild' settlers had occupied.

(iii) Substratum choice (small slates)

The vast majority of settlers were found on the slates biofilmed in FFSW + adults + algae (Table 10) and all the early settlers were on these slates. Chi^2 analysis of the data showed that there was a significant difference in the number of settled larvae on the five surfaces (probability < 0.05).

A few 'wild' settlers were found when the experiment was set up but only one larva settled close to the position of a 'wild' settler during the

experiment and this larva was one of the later settlers in the final 24 hours of the experiment.

Table 10: The settlement of *Pomatoceros lamarckii* larvae on surfaces biofilmed with and without the presence of adults in FFSW and running seawater: substratum choice (small slate experiments), results at 48 hours.
FFSW = fine filtered seawater. RSW = running seawater.

Replicate	FFSW + Adults+Algae		RSW + Adults		RSW only		FFSW + Algae		Clean Control	
	Pit	Slate	Pit	Slate	Pit	Slate	Pit	Slate	Pit	Slate
1	1	14	0	0	1	0	0	0	0	0
2	23	8	1	0	2	1	0	0	0	0
3	27	16	0	1	0	2	0	0	0	0
4	11	11	0	0	0	0	0	0	0	0
Totals	62	49	1	1	3	3	0	0	0	0

D) Glass biofilming experiments

Slides stained with Ruthenium Red showed the presence of both stained and unstained bacteria, indicating that some of the bacteria present were not surrounded by acid mucopolysaccharides. There appeared to be many more of these unstained bacteria on the slide which had been biofilmed in the presence of adults and food algae in FFSW than on the other slides. It was also apparent that overall there were far greater numbers of bacteria

present on the slide which had been biofilmed in the presence of adults and food algae in FFSW than on the other slides.

Slides stained with Crystal Violet again showed many more bacteria on the slide which had been biofilmed in the presence of adults and food algae in FFSW than on the other slides (Table 11). The bacteria on this slide included great numbers of rod-shaped bacteria *circa* 2μm. in length. Very few of these rod-shaped bacteria were found on the other slides.

Table 11: Mean number of bacteria (± SD) in $10\mu m^2$ on glass slides biofilmed with and without adult *Pomatoceros lamarckii* in fine filtered and running seawater.
FFSW = fine filtered seawater. RSW = running seawater.

FFSW + Adults + Algae	RSW + Adults	FFSW + Algae	RSW Only
60.4 ± 11.9	14.8 ± 4.4	2.29 ± 1.5	15.3 ± 6.2

There was very little difference in bacterial coverage of the slides biofilmed in running seawater with and without adults but bacteria on the slide biofilmed in FFSW + algae were very sparsely distributed.

DISCUSSION

A dictionary definition of gregariousness is "Association in flocks and herds: growing together but not matted (bot): fond of the company of others." Crisp (1990), in relating to the settlement of barnacle larvae, modifies this definition and states "Gregariousness implies that the probability of cyprid settlement is increased by the presence of one of its own species, by part of one of its own species or by an extract therefrom". If we take this latter definition to be applicable to invertebrate larvae in general then it can be said that *Pomatoceros lamarckii* larvae are indeed gregarious. However, if the term 'gregarious settlement' implies that larvae will settle close to each other then it cannot be said with any certainty that *P. lamarckii* larvae exhibit gregarious settlement. Although *P. lamarckii* larvae have, during the present laboratory study, frequently been observed to settle in very close proximity to each other there have also been many occasions where subsequent settlers have settled, in terms of larval dimensions, a considerable distance away from the initial settler(s). It can be said, however, that when an adult is present it is only on very rare occasions that none of the initial settlers are to be found on the adult tube and it has been convincingly demonstrated that the presence of an adult on a substratum makes that substratum far more attractive to settling larvae.

Gregariousness has obvious advantages for a sedentary invertebrate larva. Selection of a settlement site is a crucial phase as once the larva is attached to the substratum the process is irreversible and an unfavourable site in an unsuitable habitat may result in the premature death of the animal. Knight-Jones (1951) states "Gregariousness... is probably a general feature of the settlement behaviour of planktonic larvae, helping them to find suitable habitats, to maintain old breeding stocks and to form new ones."

The presence of healthy adults of the same species on a prospective site is a sure indication that the site is suitable for that particular species. Knight-Jones (1951) gives the location of 'safe sites' where adult populations exist as one of the reasons for gregariousness. It does not follow that the presence of newly settled juveniles of the same species is an indication of a suitable settlement site in the long term. If the initial settlers, for whatever reason, settle in an unsuitable situation, a gregarious response to other juveniles could be disadvantageous to later settlers. It would therefore appear logical that any gregarious response should be to the presence of an adult. The settlement behaviour of *Pomatoceros lamarckii* larvae adds support to this hypothesis. The presence of an adult results in enhanced settlement but the presence of newly settled juveniles does not.

During the period of larval dispersal in the plankton the pressure to find a suitable settlement site will increase with increasing age of the larvae and the chances of success are enhanced by the number of sites which can be visited. Butman (1984) suggests that larvae may swim vertically down to test the substratum and then vertically up to be advected downstream by the current. The undulating swimming motion of competent larvae observed during the present study could thus be a strategy which, in the wild, enables the larva to investigate a large number of spatially separated, shallow water locations reasonably quickly.

The result of introducing *P. lamarckii* larvae to substrata bearing adult *P. lamarckii* (Table 1) confirmed the observations of Moat (1985) and additionally showed that both the adult tube and surrounding slate substratum were highly attractive to settlers.

Close investigation of 'attractive' substrata by larvae was conducted with the ventral surface of the head region in contact with the substratum rather than the apical cilia. These cilia were only occasionally brought into

contact with the substratum, usually during the initial searching phase, when the larva adopted a head downward position. This observation suggests that these apical cilia do not play a major part in the selection of a settlement site. Additionally, Moat (1985) found that the rootlets of the apical cilia of *Pomatoceros triqueter* had no direct connection with nerve cells, although they were located in the vicinity of nervous tissue.

It has been noted that tail attachments to the substratum are made from time to time as previously reported by Føyn & Gjøen (1954) and Moat (1985) but these tail attachments are invariably temporary. Such temporary attachments have also been observed during the settlement of *Spirorbis spirorbis* larvae (Nott, 1973). Observations during the present study suggest that the role of the tail attachments is to help stabilise the larva and allow it to flex the anterior part of the body sharply at right angles to the substratum preceding the moment of attachment. It would appear that this sharp flexion of the body triggers, by abruptly tensioning the ventral shield epithelium, the explosive release of the large pad of mucus which forms the permanent attachment. Tail attachment alone would not allow this movement to take place, the posterior segments must also be attached and it is most probable that the mucus for this attachment comes largely from the ventral mucous glands of the posterior segments. When manipulating competent larvae during the present study they proved to be very sticky and would often adhere to the eyelash with which they were being manipulated. This stickiness suggests that larvae seeking settlement sites already have at least a superficial coating of mucus prior to settlement which would assist the temporary attachment.

The mucus released at the time of settlement is thought to be a sulphomucopolysaccharide (Hedley, 1956). It is very clear and consequently extremely difficult to see under the light microscope while settlement is actually taking place. The fact that there is such a pad of

mucus is only revealed by staining for light microscopy or viewing in the SEM. There can be little doubt, considering the location of the mucus pad (Fig. 6), that the bulk of this mucus is released from the abundant mucous glands of the ventral shield epithelium.

The rotational and longitudinal movements which follow settlement are necessary to shape the primary mucous tube. Formation of this primary tube by the rotary movements is almost certainly achieved by the abdominal and thoracic chaetae which, as the animal rotates, will catch in the mucous pad and drag the mucus around the body of the animal in a very similar way to that described by Nott (1973) for the tube formation of *Spirorbis spirorbis*. As the animal makes a 360° rotation in each direction mucus will be dragged completely around the animal at each rotation and re-joined to the mucus pad at the other side, quickly forming the tube. The purpose of the longitudinal body movements of the newly settled animal is not quite so obvious. These movements will ensure that the new tube is kept open while it hardens and probably also serve to spread the initial mucous pad over a greater longitudinal distance, thus increasing the length of the primary mucous tube.

Segrove (1941) established that when the contents of the anal vesicle were released the posterior end of the animal assumed a more rounded appearance and the anus returned from the dorsal surface to its original position at the posterior end. It was observed during the present study, that during the formation of the first semi-opaque tube it could be seen that the posterior end of the animal was often still pointed, indicating that the vesicle was still at least partially full of mucus. At this stage the tube was normally still open at the posterior end. As the animal grows and produces a calcareous tube any damage to the posterior end of this tube is quickly repaired with a calcareous end-plate (Fig. 3, Chapter 3.). Such damage forms a major weakness in the animal's defences, allowing easy access to

predators such as the Green Leaf worm, *Eulalia viridis* . In the course of the present study this worm, which often inhabits old *Pomatoceros* tubes, has been seen to attack exposed *Pomatoceros* species adults when their tubes have been broken open to obtain gametes for fertilisation. When the juvenile tube becomes sufficiently long to accommodate the whole animal it is possible that the mucus from the anal vesicle is used to form an end-plate as a first defence against such predators.

Faouzi (1931) and Thomas (1940) both described the base of the *Pomatoceros* species tube as incomplete, the substratum itself serving to complete the tube, but Thomas (1940) did also note that the tube could be complete in some circumstances. In fact the base of the tube is complete but is not completely calcified, often consisting solely of clear mucus, thus giving the impression of being incomplete. The presence of this mucous base can easily be demonstrated by sliding a tube off a piece of glass upon which it has been allowed to grow. The apparently open base cannot be penetrated by a bristle or other light probe thus indicating the presence of the clear, mucous sheet. When the tube is raised clear of the substratum, as can occur in conditions of overcrowding, the animal is able to rotate fully with the result that a complete calareous tube is formed.

The cavities in the thickened sides of the tube at the base which Thomas (1940) suggested were probably to economise on material can clearly be seen if the animal is grown on glass (Fig. 10). It can be seen that, as the animal and tube increase in size, the cavities show a corresponding increase. It has been noted during the present study that these cavities are very closely related in size and spacing to the strong chaetae on the thoracic region of the animal. Observations of animals induced to inhabit artificial, glass tubes (See Appendix 1) have shown that these chaetae are used as an aid to movement along the tube and that animals grown in glass tubes do not move as swiftly as those in natural

tubes until some natural tube has been added to the end of the glass tube. The mechanism of the formation of the basal cavities is not entirely clear but it appears that they are formed as a result of the action of the thoracic chaetae on the soft, newly formed tube and that their purpose is to provide traction for the chaetae as the animal moves back and forth in the tube.

The settlement behaviour of other invertebrate larvae, (Knight-Jones, 1953; Crisp & Meadows, 1962, 1963; Wilson, 1970; Cameron & Hinegardner, 1974; Chia, 1978; Morse, 1990) suggests that a common cause of gregarious settlement in invertebrate larvae is a chemical stimulus produced by the respective adult and adsorbed onto the substratum. When, early in the present study, it became obvious that *Pomatoceros lamarckii* larvae were settling gregariously the most promising and logical starting point in the search for the cause of this gregariousness appeared to be to attempt to isolate, at least in a crude form, such a chemical stimulus.

Exhaustive attempts to isolate a chemical stimulus in a variety of solvents and from various parts of the animal, tube, larvae and algal food sources have met with little success. The most promising results were obtained from algal extracts and extracts of homogenised larvae. However, these results were not consistently repeatable and, in the light of later work, it was clear that they could also be explained in terms of the effects of biofilming.

A possible cause of the failure to isolate a chemical stimulus could have been that the chemical was degraded, destroyed or lost during the process of extraction, e.g. digestive enzymes from digestive tract of the complete worm could have adversely affected any chemical attractant which may have been present. Attempts were therefore made to transfer an attractant to experimental surfaces by swabbing, smearing etc. as previously detailed. None of these attempts proved successful in terms of larvae being attracted to the treated surfaces but it was noticed that, where biofilmed surfaces

were used as the experimental substrata, larvae settled readily on these otherwise untreated surfaces.

The results up to this point suggested that it was fairly unlikely that gregariousness in *Pomatoceros lamarckii* was due to a chemical stimulus but further attempts to demonstrate the presence of a 'settlement substance' were made. The majority of these experiments yielded no positive results but two experimental approaches were worthy of note.

Larval conditioning of pits in slate substrata proved to be inconclusive and, in those experiments where the experimental surfaces were used in two consecutive experiments, the positions of settlers in the second experiment showed no correlation with the positions of the settlers in the first one. This result supports the hypothesis that larvae will not necessarily benefit from settling on substrata recently settled by other larvae of the same species. The results also showed that biofilming was an important factor in settlement as the clean controls were not settled but all biofilmed surfaces were. This disparity between settlement on clean and biofilmed surfaces was demonstrated many times, establishing beyond question the role of biofilming in the settlement process.

The exhalent stream experiments showed that no clear relationship existed between the exhalent stream from the adult and the settlement pattern of the larvae but the experiments utilised substrata which had been biofilmed both in and without the presence of adult *P. lamarckii* . It was clear from the results that substrata biofilmed in the presence of adults were more attractive than those biofilmed without.

The possibility of the existence of a chemical attractant now seemed remote and the evidence was beginning to suggest strongly that biofilming had a major role to play in settlement. This suggestion was further strengthened by fieldwork carried out at Dinas Dinlle, a high energy environment, storm beach situated on the Lleyn Peninsula (Chapter 6).

Previous fieldwork, carried out in the Menai Strait, had almost always shown adult *Pomatoceros* species and juveniles in close proximity on the same stones. Examination of stones at Dinas Dinlle showed large numbers of juveniles gregariously settled on stones which held no adults and which showed no traces of the remnants of old adult tubes (Fig. 11).

It was also abundantly evident that, despite no chemical attractant having been found, the presence of adults strongly influenced settlement. Either a chemical attractant did exist but the methods used had failed to extract it, or the adults influenced settlement in some other way, possibly by creating conditions suitable for the growth of a biofilm which was specifically attractive to *Pomatoceros* species larvae.

A question which must constantly exercise research workers is that of how many repeats of an experiment should be made when that experiment produces negative results. (It can be argued that no result is negative but the term as used here refers to those results which do not support the hypothesis which is being tested). A little time spent working with larvae soon shows that settlement can be very unpredictable. An experiment carried out with a poor batch of larvae or one in which the larvae are not actually competent may result in a totally misleading outcome. How often should one repeat that experiment in the hope of obtaining a positive result? It is far easier to demonstrate that something exists or is true than to show without doubt that something does not exist or is untrue. Such is the case with the chemical attractant work reported here. Further experimentation may yet show that a chemical attractant is indeed produced by adult *P. lamarckii* but time constraints and strong evidence to the contrary directed that further research should be devoted to examining the effects of different types of biofilming on larval settlement.

An early comparison of slates biofilmed in the presence of adults and food algae in FFSW (adult/FFSW biofilmed slates) and slates biofilmed in

Figure 10.

Light micrograph of the underside of a *Pomatoceros lamarckii* tube which has been formed on glass, showing the cavities in the sides of the basal area. The base of the tube appears to be incomplete but it can be seen that the clear, mucous sheet, which completes the base has become lightly calcified in the posterior third of its length, giving it a milky appearance (arrow). Scale bar...750 μm.

Figure 11.

A pebble from the beach at Dinas Dinlle, Lleyn Peninsula. The majority of larvae have settled gregariously at the level of the sand surface and are growing upward. At least three different batches of settlers may be distinguished by tube size. Note also the isolated settlers and small group of settlers (arrows).
Scale approximately life-size.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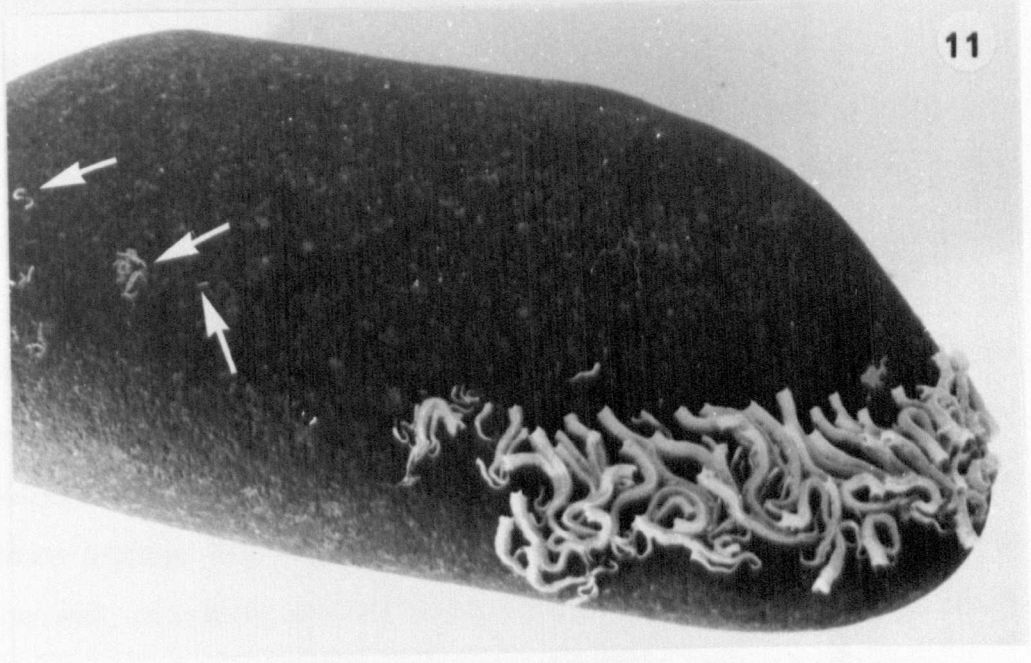

11

running seawater only (RSW biofilmed slates) showed an apparently strong preference for the adult biofilmed slates. However, limitations in the biofilmed substrata available when the experiment was run meant that the RSW biofilmed slates had been biofilmed for a shorter period than the adult biofilmed slates. Therefore the result could have been due to differences in the length of time for which the slates had been biofilmed or to the fact that one lot of slates had been biofilmed in FFSW and the other in RSW rather than to the presence or absence of adults. The design of the later, definitive biofilming experiments overcame these problems.

A curious fact concerning the settlement of *Pomatoceros lamarckii* larvae is that the vast majority of larvae in any competent batch will, in laboratory conditions, perish rather than settle on an unattractive substratum. In terms of survival of the species this behaviour would appear to be disadvantageous, as it is surely preferable to settle on any hard surface with the possibility that it might prove to be a suitable habitat rather than not to settle at all. Two points need to be borne in mind; laboratory conditions are greatly different from those which appertain in the wild and, secondly, to maintain the *status quo* only two larvae from a normal batch of around three to four thousand need to survive to adulthood. There is no way of knowing whether *P. lamarckii* larvae respond to unattractive substrata in the same way in the wild as they do in the laboratory but if only two or three larvae from a batch will ultimately settle an unattractive substratum perhaps this rate of settlement is sufficient. Predation in the plankton will, of course, ensure that very few larvae will actually reach competence, whereas in the laboratory the majority of larvae survive.

The substratum acceptability experiments assessed the individual attractiveness of a variety of biofilmed surfaces when there was no choice available. In view of the observed unwillingness of *P. lamarckii* larvae to settle an unattractive surface the results of these experiments can be

considered to be a good indication of the attractiveness of a particular biofilm. The results of these experiments showed a clear preference for surfaces which had been biofilmed in the presence of adults but there was little to choose between adult/FFSW biofilmed slates and adult/RSW biofilmed slates. Overall the preference was for the former but this result represented a negligible difference of seven larvae in a total of 600. However, if the result represented a real difference in preference for the biofilm one would expect similar choices to be made when the larvae were given free choice between the available surfaces.

When this choice was given (larval choice experiments), although the order of preference was the same, a clear difference between the adult/FFSW biofilmed slates and the adult/RSW biofilmed slates was evident. There was a further clear difference between the adult/RSW biofilmed slates and the remainder of the choices available.

The final set of larval choice experiments, using small slates, proved to be a convincing confirmation of the previous results with 93% of the settlers to be found on the adult/FFSW biofilmed slates.

It is quite clear that slates biofilmed in the presence of adults provided the most attractive substratum. At first glance it may appear strange that the adult/FFSW biofilmed slates were more attractive than the adult/RSW biofilmed slates but if we accept the hypothesis that the attraction is primarily due to specific bacteria and/or their exudates within the biofilm a satisfactory explanation can be proposed. Initially there would be many more bacteria in the running seawater than in the FFSW but the build up of faeces and other waste products in the static FFSW would quickly lead to high bacterial densities. The adult/RSW biofilmed slates were in a much greater volume of water initially and this water was constantly being replenished, leading to a slower overall build-up of bacteria on those slates. It should be remembered that the substratum choice experiments showed

the adult/FFSW biofilmed slates and the adult/RSW biofilmed slates to be very similar in their attraction to settlers when no other alternative was available. When given a choice the larvae chose the more attractive of the two options.

The low settlement on FFSW + food algae biofilmed slates and the RSW only biofilmed slates lends further support to the hypothesis. Slates biofilmed in FFSW + food algae could be expected to have very few bacteria of any kind and the RSW only biofilmed slates faced problems of increased volume of water and no adult *Pomatoceros lamarckii* present to encourage the growth of the right type of bacteria.

More support for the hypothesis was forthcoming from the examination of the bacterial slides. Once stained it was even evident to the naked eye that the bacterial film on the adult/FFSW biofilmed slates was more dense than on any of the others. What became evident from examination under a microscope was that not only was this film more dense but the adult/FFSW biofilmed slates had a large number of bacteria which were absent, or present only in low numbers, on the other slides.

Two other interesting points arise from the final series of experiments. Settling larvae, with only one exception, never settled near the places from which wild settlers on the RSW slates had been removed. On one occasion 15 wild settlers were removed prior to the experiment from a pit in a slate which had been biofilmed in RSW only. Clearly this pit was attractive to settlers but no larvae settled in or near that pit during the course of the experiment and only four settlers were found on the whole slate. The biofilming on the adult/FFSW biofilmed slates was obviously considerably more attractive than that on the RSW only biofilmed slate, but this observation also reinforces the earlier point that larvae are not necessarily going to settle close to other juveniles with no mature adult present. The one exception mentioned was a single, late settler which settled near to the

position of a wild settler. As this larva was a late settler it suggests that the choice of settlement site was unconnected with the position of the wild settlers as earlier settlers would have selected the site if the wild settler had provided a particularly attractive stimulus.

The second point concerns the pits. Slates with flat-bottomed pits were used because it had been found in initial experiments that this shape was conducive to settlement. Larvae appeared to find the angle between the side and the base of the pit a particularly attractive settlement site. The pits were well settled during the final series of experiments but only where the biofilm was attractive, demonstrating again that settlement was motivated primarily by the biofilm and not by the topography of the substratum.

Chapter 6

Field Observations of *Pomatoceros lamarckii*

INTRODUCTION

Results which are obtained in the laboratory should not be viewed entirely in isolation, as they do not necessarily reflect the natural behaviour of the organism under investigation. Whenever possible such results should be considered in the light of what takes place in the field and should thus ultimately lead to a better understanding of the organism. In order to correlate the experimental findings of the present study with the behaviour of *Pomatoceros lamarckii* in its natural surroundings, field observations were carried out in the spring and summer months of 1990 - 1992, when optimum settlement of *Pomatoceros* species larvae is to be expected. There were two main objectives:

- To determine whether *Pomatoceros* species larvae preferentially settle close to adults.
- To determine whether settlement was greater on the upper surfaces of substrata or on the under-surfaces where these surfaces were accessible.

The field work necessary to attain these objectives was carried out at three locations where *P. lamarckii* is known to be plentiful. These locations offered a variety of environmental conditions from the strong

tidal race of the Menai Strait to the storm beach of Dinas Dinlle and the sheltered beach at Moelfre.

MATERIALS, METHODS AND RESULTS

Random Sampling 1990

Random sampling of stones in the Menai Strait was carried out during the summer of 1990 in the vicinity of the Telford road bridge in order to determine the first objective. On stones which are densely populated by *Pomatoceros* species it is often difficult to establish the original point of settlement of a particular animal, therefore only those juveniles for which the point of origin could clearly be seen were counted. A juvenile was defined as an animal with a tube length <10mm. (not necessarily a recent settler) and was considered to be on or close to an adult if its point of origin lay at a distance of <5mm. from the adult tube. No attempt was made to distinguish between animals on upper and lower surfaces of the stones at this stage. 70.5% of all juveniles which conformed to the above criteria were found to be on or close to an adult tube (n=173). This finding supported the results obtained in the laboratory (Chapter 5).

Raft Experiments 1991

In May 1991 six roofing slates were fastened to wooden panels and suspended vertically from a raft situated in the Menai Strait just east of the town of Menai Bridge. The slates were approximately 180 x 100 x 5mm. The overall size was unimportant as a 75 x 75mm. experimental area was marked out on each slate within which settlement was recorded. Adult *P. lamarckii,* initially attached to the slates using a cyanoacrylate adhesive and

then allowed to grow naturally onto the slate, as described in Chapter 5, were situated at the centres of three of these experimental areas.

The slates were inspected weekly for settlers until the end of July when the panel was lost from the raft. A new panel holding four slates, with experimental areas delimited as described above, was quickly substituted for the lost panel. No adults were attached to these slates due to time constraints. The experiment was terminated in mid-September.

Settlement in the experimental areas was poor (Table 1). Only one larva settled in contact with an adult (on slate 6). This isolated settler was found on the first inspection. Most of the settlement occurred in mid-July. This settlement appeared to be random, with only one other settler close to an adult (slate 3). The slates were heavily colonised by other organisms, mainly barnacles and algae suggesting that *Pomatoceros lamarckii* larvae were not present in any numbers in the vicinity of the raft. Despite the poor settlement it can be seen that 70% of the larvae settled on slates with attached adults.

The replacement slates attracted only two *P. lamarckii* larvae by the time the experiment was terminated in September.

Table 1: The settlement of *Pomatoceros lamarckii* larvae on slate substrata, with and without attached adults, suspended in the Menai Strait between May and July 1991.

Slate 1 no adult	Slate 2 with adult	Slate 3 with adult	Slate 4 no adult	Slate 5 no adult	Slate 6 with adult
0	0	11	3	4	5

Settlement Experiments 1992

As the raft location appeared to be unsuitable in terms of larval density it was decided to repeat the experiment, commencing in May 1992, with similar slates placed intertidally in the Menai Strait close to the Telford road bridge, where large populations of *Pomatoceros lamarckii* are to be found. This latter location had the apparent further advantage of being accessible on foot for at least one hour either side of low water.

Six slates were prepared as described for the raft experiment and tied in pairs (consisting of one slate with and one slate without an attached adult) to three sheets of 10mm., heavy duty plastic mesh. The sheets of plastic mesh, with attached slates experimental surface upward, were then placed in easily identified locations on the rocky bed of the Strait and weighted down with large boulders. In addition to these large slates, five 50 x 50 x 3.5mm. slates, three carrying attached adults, were also placed experimental surface upward in the same location.

This experiment could best be described as a disaster. Despite being inconspicuous and well weighted down, all but one of the slates disappeared in quite a short time. This disappearance was attributed to the activities of professional crabbers and other people searching for bait and/or fishing tackle at low tide. The one remaining slate was attached to plastic mesh and originally had no attached adults. On examination the upper surface had heavy algal fouling and the lower surface had five *Pomatoceros* species settlers.

In addition to the obvious lesson to be learned about siting experiments in locations easily accessible to the general public, it is also worth noting that any results of studies involving upper and lower surfaces of stones in such an area would be suspect as one could never be entirely sure whether an upper surface as found was the upper surface at the time of settlement.

Random Sampling 1992

1. Menai Strait.

Random sampling was carried out in the Telford road bridge area to ascertain the relative numbers of adult and juvenile *Pomatoceros* species on the upper and lower surfaces of stones before it became apparent that the area could be subject to considerable disturbance. Adults were again defined as those in tubes >10mm. in length except very old tubes and broken fragments. Juveniles were taken to be animals with tubes <10mm. in length. 'On or close to an adult' was taken to be <5mm. from the adult tube and a distance >5mm. was considered to be 'away from an adult'. Upper and lower surfaces were considered in terms of settlement. Some animals settle on one surface and then grow onto another. In this study the surface on which the animal settled was recorded. Selection was not entirely random in that reasonably flat stones, where settlement was largely restricted to a choice between top and bottom surfaces, were selected. The results of this survey are given in Table 2. The problem presented by stones with definite sides was addressed in later sampling carried out at Dinas Dinlle and Moelfre.

It can be seen from Table 2 that most of the juveniles settled away from adults. This result is the reverse of that obtained from sampling the same area in 1990. The majority of adults were found on the upper surfaces of the stones.

2. Dinas Dinlle Beach.

Dinas Dinlle, situated on the Lleyn Peninsula, is a sandy, exposed storm beach with isolated patches of boulders and pebbles which are exposed at low tide. Random sampling was carried out on this beach using

Table 2: The distribution of adult and juvenile *Pomatoceros lamarckii* on stones from the inter-tidal area of the Menai Strait close to the Telford road bridge. The totals represent the numbers of animals found on 113 stones comprising three random samples. **FA** = from adult.

	Upper Surface			Under-surface		
	Adults	Juveniles		Adults	Juveniles	
		< 5mm FA	>5mm FA		< 5mm FA	>5mm FA
Total	343	56	125	185	20	112

the same criteria as stated for the sampling done in the Menai Strait. Very few live, healthy adult *Pomatoceros* species were to be seen, therefore most of the figures recorded for adults are based on old tubes. Where doubt as to actual numbers occurred the lower figure was taken. Fig. 1 illustrates a possible source of error; the parallel pieces of tube could be from one animal or from two and without further evidence one animal would be recorded.

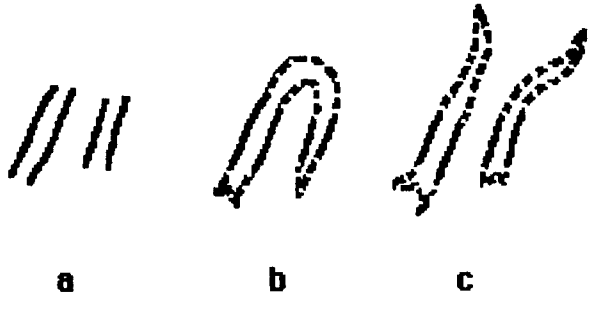


Fig. 1: Pieces of old *Pomatoceros* species tube (**a**) could be from one animal (**b**) or two separate animals (**c**).

Two samples were taken, one of which included pebbles with definite sides. The results are shown separately in Tables 3 and 4.

Table 3: The distribution of adult and juvenile *Pomatoceros lamarckii* on the upper and under-surfaces of pebbles from Dinas Dinlle beach. The totals represent the numbers of animals found on 31 pebbles. **FA** = from adult.

	Upper Surface			Under-surface		
	Adults	Juveniles		Adults	Juveniles	
		<5mm FA	>5mm FA		<5mm FA	>5mm FA
Totals	140	28	25	13	4	361

It can be seen that the vast majority of adults were found to have settled on the upper surfaces of the pebbles and the vast majority of juveniles on the under-surface. To put this finding into context; 230 of the juveniles found were on the underside of one pebble which, interestingly, carried no adults and was well buried in the sediment. The majority of juveniles were found away from adults.

In some cases the juveniles on one surface were so great in number and so closely associated that estimates of the number present had to be made. Totals involving estimated numbers are indicated by the ≈ symbol. So far as could be ascertained most of these juveniles were alive.

Again, most adults were found to be on the upper surface and the majority of juveniles on the lower surface. More juveniles settled away from adults than close to them. With only one exception, where large aggregations of

juveniles were found no adults were present on the surface where settlement took place or on other surfaces of the pebble. Settled larvae were again found on the undersides of pebbles which were well buried in the sediment. The majority of these larvae were alive and actively tube building.

Table 4: The distribution of adult and juvenile *Pomatoceros lamarckii* on all surfaces of pebbles from Dinas Dinlle beach. The totals represent the numbers of animals found on 34 pebbles. **5 FA** = 5mm. from adult.

	Upper surface			Sides			Under-surface		
	Adults	Juveniles		Adults	Juveniles		Adults	Juveniles	
		<5 FA	>5 FA		<5 FA	>5 FA		<5 FA	>5 FA
Totals	318	≈649	≈971	143	≈354	≈1280	31	214	≈1563

3. Moelfre Beach.

Moelfre beach is a rocky beach with coarse, shell sand in pockets among the rocks. It is situated on the east coast of Anglesey and is consequently sheltered from the prevailing south-westerly winds. An offshore island affords shelter from easterly winds. One random sample consisting of 38 stones was taken from this beach (Table 5).

These distributions differed from those at the other two sites in that both adults and juveniles were fairly evenly distributed. Most adults, by a narrow margin (3.5%) were found on the upper surfaces of the stones and most juveniles on the undersides by an even narrower margin of 0.8% Juveniles which settled away from adults were in a majority of 1.6%.

Table 5: The distribution of adult and juvenile *Pomatoceros lamarckii* on stones from Moelfre beach. The totals represent the numbers of animals found on 38 stones. **5 FA** = 5mm. from adult.

	Upper surface			Sides			Under-surface		
	Adults	Juveniles		Adults	Juveniles		Adults	Juveniles	
		<5 FA	>5 FA		<5 FA	>5 FA		<5 FA	>5 FA
Totals	272	398	333	177	256	319	248	356	390

Table 6 gives a summary of all the random sampling results for all three sites..

Table 6: A summary of the results of all random sampling at all three sites, with the distribution of adult and juvenile *Pomatoceros lamarckii* expressed as percentages. **FA** = from adult.

	Upper surface	Sides	Under-surface	Juveniles <5mm. FA	Juveniles >5mm. FA
Adults	65	14	21	-	-
Juveniles	35	15	50	35	65

DISCUSSION

The experiments involving prepared slates suspended from a raft or placed in the Menai Strait provided very little useful data. The most interesting results were obtained from the random sampling carried out at the three locations. Before considering the results of this sampling and their implications a few general comments on the complexities of this apparently simple exercise will be found useful to put the findings into context.

There are many examples of the power of waves and this author has personally witnessed wave transport of boulders weighing many tons during storms on the Cornish coast. Consequently there can be no certainty about which side of a small stone is top or bottom in an area where strong currents abound and/or the substratum is subject to pounding by high energy storm waves. At all locations used in this study there was evidence (and often the presence) of people looking for bait and beachcombers searching for fishing tackle etc. Thus one cannot categorically say that an adult tubeworm spent most of its life on the upper or lower surface of a small stone from an examination of that stone unless there are other indications as to which surface has been predominantly uppermost. The shape of the stone might ensure that it is stable only when a particular surface rests on the substratum. The absence of macroalgae on a surface may usually be taken as a good indication that this surface has been the underside for some time. This latter indication is useful when looking at settlement on small stones. As juvenile *Pomatoceros* species can be aged to within a few days by their average tube length, it is relatively easy to compare the time which the juvenile has been on the stone with the time required for the stone to have acquired a complement of macroalgae.

When considering the settlement preference of *Pomatoceros* species larvae, defining specific surfaces becomes problematic. Fig. 2 illustrates this point.

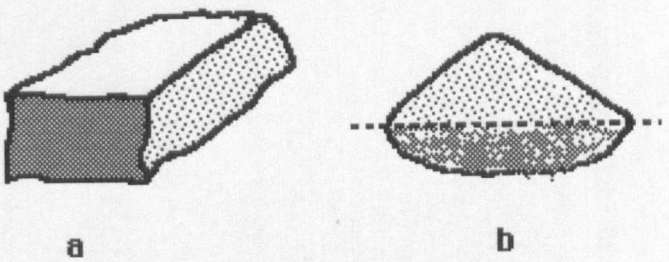


Fig. 2: A stone (**a**) may have clearly differentiated sides but a rounded pebble (**b**) does not.

The stone (**a**) presents no problems. Top, bottom and sides may clearly be differentiated. The pebble (**b**) however, is much more problematic. Is all the area above the dotted line 'top' or can some of it be considered to be sides? Are the sides just around the area of the dotted line? Could some of the area below the dotted line be considered to be sides? An almost spherical pebble presents even more problems with regard to both the definition of surfaces and what length of time a surface of the pebble remains 'top' or 'bottom'.

Whether the point at which the larva settled, or the position which the adult animal occupies is recorded will depend upon what aspect of the animal's behaviour is being examined. The actual point of settlement can often be difficult to identify, especially in areas of high settlement density, but it is normally relatively easy to determine the surface upon which the adult originally settled on stones which have well defined surfaces.

It is not possible to make accurate counts of juveniles in the field. Time constraints imposed by tidal movement prevent a detailed examination of a meaningful sample and good illumination and magnification are a prerequisite to the location of newly-settled juveniles. If samples are to be removed to the laboratory for examination a reliable way of recording the upper or lower surface is necessary. A diamond stylus was used for this purpose in the present study and was, in the main, satisfactory but it was occasionally difficult to relocate the stylus mark on hard surfaces. A possibly better, but more time consuming, method of marking would be to put the samples on a tray, carry them above high water level, allow them to dry and then mark them with paint or nail varnish.

Because of the need for microscopic examination, one cannot realistically include large rocks when sampling for the presence of juveniles. Nor can the undersides of such rocks be examined. The examination of one medium-sized boulder could take more time than would be available between exposure and re-flooding of the site. One can, however, obtain an overall impression of the distribution of adults on large boulders and can also be more certain that top, bottom and sides have existed as such for a considerable period of time.

The results indicate that although the majority of adults was found initially on upper surfaces the majority of juveniles found had settled on under-surfaces. This finding does seem rather strange but the explanation is obvious when one examines the raw data. It can be seen that the data are heavily biased by a few isolated cases at Dinas Dinlle where numbers of up to *circa* 1000 juveniles were found on the undersides of pebbles which had few or no settlers on the upper surfaces.

The most interesting aspect of the fieldwork is, in fact, the discovery at Dinas Dinlle of these heavy, gregarious settlements of larvae on stones which showed no obvious sign of living adults or old adult tubes. These

larvae were found mainly on the sides and undersides of the stones and those on the underside were often well buried in the sediment. Macroalgae growing on the stones in question showed that the upper surface as found had been uppermost for considerably longer than the period over which the larvae had settled. The sizes of the juvenile tubes found in early July indicated that there had been two distinct settlement periods, one around early June and one around the end of June. Stones taken from high on the shore were sparsely settled and there was no settlement on their undersides, the heavy settlement being confined to substrata on the low shore. This finding is entirely consistent with the lifestyle of *Pomatoceros* species, which one would expect to find in increasing numbers from the lower littoral downward. It was apparent that a considerable number of larvae settle on the sides of stones and then grow towards and onto the upper surface (Fig. 11, Chap. 5).

With the exception of the juveniles which were found to have settled beneath the sediment, the findings for Dinas Dinlle are probably consistent with what might be expected from a high energy, wave-swept environment. The act of settlement is almost certainly more difficult on the tops of stones, where there is little or no protection from wave action. Larvae which do settle on the upper surface stand a high chance of being knocked, swept or abraded off before a sufficiently strong calcareous tube can be formed. On the other hand those which settle on the sides will have some degree of shelter during and after settlement and will still easily be able to feed. Adults attached to the undersides of stones which are buried in the sediment would not be able to feed unless growing in hollows or niches which were free of sediment. Even in this situation feeding could well be difficult for a suspension feeder which relies on a steady supply of food particles.

It would appear, therefore, that the different requirements which larvae and juveniles have from adults determines the settlement pattern at Dinas Dinlle. There must obviously be sufficient space between the sand grains for the larvae to move within the sediment until a substratum suitable for settlement is encountered. Once settled the juveniles can only reach a certain size before feeding becomes increasingly difficult and only those which can grow up onto the sides of the stones will reach adulthood. Those larvae which settle at the sediment/surface interface on the sides of stones are probably in the optimum settling situation, with sheltered conditions for settlement and continued access to a food supply as growth continues. The noted tendency for settled animals to grow towards the upper surfaces of stones suggests that they are either negatively geotactic or positively phototactic, but no experimental work has been carried out in this area.

The overall results of the random sampling in the field show that the majority of larvae do not settle on or near adults. These results would not be affected by changes in the orientation of stones. However, there is little consistency between the results from different samples, e.g. the 1990 Menai Strait results showed that the majority of larvae had settled close to adults but the 1992 data for the same area gave the opposite result. The relative sparsity of adults at Dinas Dinlle suggests that mortality is high at this site, probably caused by animals being crushed or dislodged as the beach pebbles are moved around during storms.

Available evidence of the position, shape, size and algal colonisation of samples at Dinas Dinlle suggests that it is likely that the majority of samples were in the same orientation when found as when settlement took place. However, a change in orientation caused by human intervention or natural forces cannot be ruled out. For this reason it would be unwise to draw any firm conclusion concerning settlement on upper and lower stone surfaces

from the data available at present. The results should thus be seen as showing a possible trend towards better survival of those larvae which settle on the upper surfaces of mobile substrata, as evinced by the fact that 65% of all adult *Pomatoceros lamarckii* examined were found to have settled on the upper surfaces of pebbles (Table 6).

The fact that larvae in the field have been found to settle in large numbers on substrata which do not have any visible trace of adults is highly significant. This finding adds strong support to the hypothesis advanced in Chapter 5 that the larvae primarily settle in response to a biofilm. The specificity of this biofilm may explain why one stone should have a large population of newly settled juveniles while another in the immediate proximity has none.

The results of the sampling work at the three sites have shown very varied patterns of settlement and have proved sufficiently interesting to suggest that they might form the basis of some more detailed study at a future date. In particular the problems posed by movement and changes in orientation of loose substrata need to be addressed (probably by a programme of long-term marking and observation of selected stones) before really meaningful data on preferential settlement of such surfaces can be obtained.

Chapter 7

Some Observations on the Adhesion of the Calcareous Tube of *Pomatoceros lamarckii* and the Holdfast of *Laminaria digitata*

INTRODUCTION

The calcareous tubes of the adult sedentary tubeworm, *Pomatoceros lamarckii* (Polychaeta: Sabellida: Serpulidae) are commonly found intertidally on the rocky shores of the British Isles. *Pomatoceros* species are important fouling organisms and readily colonise man-made structures, from which they are not easily dislodged. Tube growth can be rapid, varying from 16 -70mm. in the first year and in summer may be 10 times that in winter (Nelson-Smith, 1967).

There is no information in the literature which deals in any meaningful way with the adhesion of *Pomatoceros* species. Tube formation, which is indirectly related to the adhesion of the animal, has been investigated by several workers: Faouzi (1931) made an early study of *P. triqueter* tube formation in which he noted that the tube was incomplete, the substratum itself forming the base. He also determined that the dorsal keel, which is a characteristic feature of all *Pomatoceros* species tubes was produced by a prominent mid-ventral fold in the collar. Robertson and Pantin (1938) gave a short account of factors affecting tube formation and observed that the animal was apparently able to produce calcium carbonate in artificial seawater in the absence of a dietary source of calcium. Thomas (1940)

noted that the ventral area of the tube can be complete in some circumstances.

Swan (1950) made the first in-depth study of *Pomatoceros* species tubes. Using radio-active strontium as a tracer he found that calcium is partially taken up directly from seawater into the tube without intervention by the worm. He also found that at least part of the calcareous material is secreted to the outside of the animal by the major subcollar glands. Hedley (1956) implied that the direct take-up hypothesis is incorrect; he gave no reason for this implication but demonstrated the existence of the exocrine tubulo-racemose glands responsible for the production and secretion of calcium carbonate. However, Neff (1971) reaffirmed the view that serpulids are able to obtain some of the calcium required for tube construction directly from seawater. Simkiss & Wilbur (1989) stated that when the decalcified acid mucopolysaccharide matrix of a *Pomatoceros* tube was kept in a solution containing calcium carbonate and bicarbonate ions the matrix became re-mineralised. This finding lends strong support to the direct take-up hypothesis.

Laminaria digitata (Phaeophyceae: Laminariales: Laminariaceae.), the common kelp, is a North Atlantic species which extends from north of the Loire, south of Brittany, to the Kara sea and also round to Spitzbergen, Iceland, East Greenland, Newfoundland, Nova Scotia and as far as Cape Cod (Kain, 1979). Around the British Isles *L. digitata* is to be found along rocky shores in a belt extending from the extreme lower littoral to a depth of at least two metres below mean low water. The mature sporophyte consists of a holdfast, a long, flexible stipe and a blade which tends to split into strips as it becomes older. The holdfast, which is made up of a number of root-like haptera, provides a very secure hold on solid substrata, including man-made structures, where it is found in many of the same situations as *P. lamarckii*. Blade growth is from a basal meristematic

region which allows the plant to keep pace with the constant wear on the blade tips.

So far as can be ascertained, very little work has been done on the adhesion of *Laminaria digitata* and few papers which have any bearing on the topic have been uncovered. Yendo (1911) described the primary holdfast of very young sporophytes as 'disc-shaped' and also described the way in which haptera and rhizines (*sic*) develop to form the secondary holdfast. Delf (1932) carried out pulling tests on stipes of *L. digitata* but not on the holdfasts, so her results have little to contribute to the knowledge of the adhesion of the plant. Davies *et al.* (1973), in an ultrastructural study of the epidermal cells in the apical meristem region of the secondary haptera of *Laminaria* species, found that the dominant feature is their active secretory role in producing vast quantities of polyphenolic substances. Tovey & Moss (1978) studied the production of rhizoids by cut sections of *L. digitata* haptera and the way in which the rhizoids attach themselves to a variety of materials. The major conclusion was that the cells of the rhizoids grew to fill every microscopic variation in surface texture, building up an exact impression of the profile of the substratum. The rhizoids secreted abundant mucilage which acted as a bonding agent both to adjoining rhizoidal cells and to the substratum.

Finally, Moss *et al.* (1981) looked at the colonisation by kelps on oil rig platforms. They suggested that such colonisation results from the drifting of young sporophytes. They found that the rhizoids appear to have no definite outer boundary to the cell walls and that mucilages appear to flow into the water and have the ability to 'set' after flowing into the irregularities of the substratum.

Although there is a lack of information on the adhesion of *L. digitata* some relevant work has been done on other algae.

Hardy & Moss (1979) examined the effects of the substratum on the morphology of the rhizoids of *Fucus* germlings and found that rhizoids were shorter and stouter on rough substrata and that a more secure attachment resulted from the greater surface area. They noted that mucus conforms to the surface shape and that rhizoids will penetrate weaknesses to increase their hold.

Fletcher & Baier (1984) found that critical surface tension affected the growth of rhizoids of *Enteromorpha intestinalis*. On substrata with a high critical surface tension the alga developed a compact, discoid rhizoidal base with short, much branched and tightly adjoined filaments, whereas on substrata with a low critical surface tension long, outwardly spreading free filaments developed. Plants grown on high surface energy substrata were difficult to remove but those grown on low surface energy substrata were easily removed by brushing. Pulling tests to establish relative tenacities, however, were not made.

MATERIALS AND METHODS

Microscopy

Light microscopy was carried out with a Leitz Dialux compound microscope and a Wild M3Z stereo microscope. Photographs were taken with a Leitz Orthoplan photomicroscope using 35mm. film.

Specimens were prepared for scanning electron microscopy (SEM) by fixing in 2.5% glutaraldehyde in seawater, rinsing in distilled water and then dehydrating through an ascending ethanol series and finally into acetone before critical point drying from liquid carbon dioxide in a Polaron E3000 critical point drying apparatus.

Dried specimens were attached to aluminium SEM stubs by means of double-sided 'Sellotape'. Finally, the specimens were coated with gold using a Polaron E5000 sputtering system before viewing in a Cambridge Stereoscan S120 scanning electron microscope operated at 10Kv.

Preparation of materials for measurement of tenacity

Flat stones with attached *Pomatoceros lamarckii* adults were collected from the Anglesey shore of the Menai Strait to provide samples from the wild. *Laminaria digitata* sporophytes were also collected from this location. Fertile sporophyte blades containing sori were obtained and whole young sporophytes were taken for transfer to experimental surfaces.

Welsh slate was used as the substratum for the majority of the tenacity tests for both *P. lamarckii* and *L. digitata.* Pieces of split slate, obtained from the Dinorwic quarry, were used to provide a naturally rough substratum. In the tests on *P. lamarckii* tubes and *L. digitata* haptera attached to smooth surfaces, slates cut to 20 x 50 x 3.5mm. and 50 x 50 x 3.5mm. were used. In order to ensure that substrata were as near identical as possible, the slates were split from one block and cut to the above sizes by Inigo Jones Slateworks, Pen y Groes, Gwynedd. The slates were finally rubbed down by hand to a uniform finish (on the test surface only) on 320 grit wet and dry abrasive paper and then ultrasonically cleaned. Wood was used as a substratum for one *P. lamarckii* test only. The resident adult had been transferred to the wood as a recently settled juvenile and allowed to grow to maturity.

P. lamarckii tubes were grown onto laboratory-prepared surfaces by prising a tube, complete with the animal within, off a stone obtained from the Menai Strait and attaching it to the required surface with a small amount of cyanoacrylate adhesive (Loctite Super Glue 3). This adhesive

provides an effective bond within seconds, is non-toxic to *Pomatoceros* species and allows the tube to be attached to the substratum in any desired position. The prepared surface and attached animal was then maintained in a tank of running seawater and normal tube growth resulted in the tube becoming permanently attached to the experimental surface after a few days. Surfaces prepared in this way were continuously immersed until the permanently attached tube was sufficiently long for experimental purposes. *P. lamarckii* tubes from the Menai Strait, attached to suitable stones, were also used in tenacity tests to provide a comparison with the test substrata.

Due to its tapering cross-sectional shape and convoluted nature, a *P. lamarckii* tube presents problems in attaching a loop which can be connected to a strain gauge and will transmit the strain evenly over the base of the tube. Two methods were devised to overcome these problems:

1. A small length of stainless steel wire could be inserted into the central depression of a coiled tube and secured with quick setting epoxy resin adhesive (Fig. 1). This method was of limited use as it depended on having tubes of the correct shape but it was the only method which allowed a complete tube to be pulled. It was unnecessary to remove the animal from the tube when using this method of attachment. Before the tube was pulled on the strain gauge the shape of its basal perimeter was carefully marked on the substratum using a fine needle. This procedure allowed the surface area of the base of the tube to be measured even if the tube itself shattered during the pull.

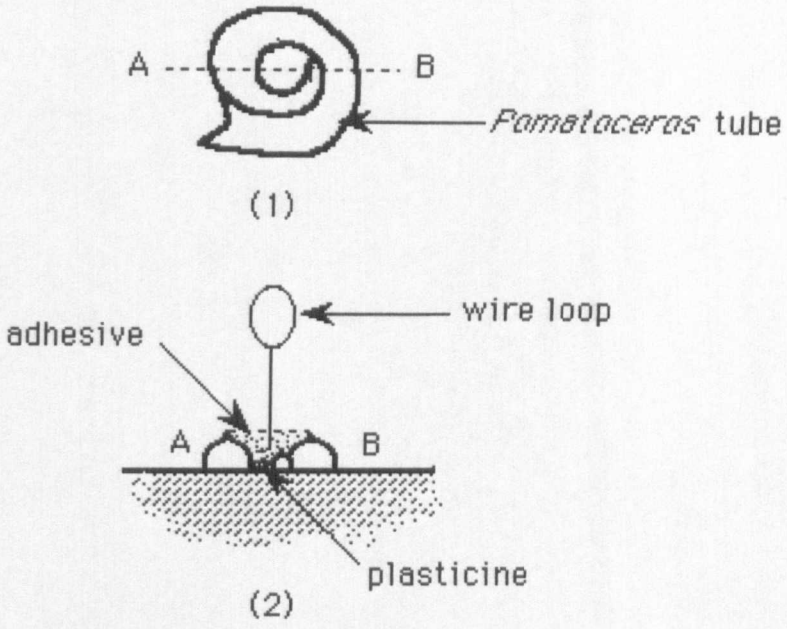


Fig. 1: The method of attaching a wire loop to a coiled *Pomatoceros lamarckii* tube. (1) Plan view of tube. (2) Section A - B showing the wire *in situ*. Note that a small piece of Plasticine is used to prevent adhesion between the adhesive and the substratum.

2. Animals were removed from selected tubes by breaking pieces off the posterior ends of the tubes until the tubes were sufficiently short to allow the animals within to be pushed gently backwards and out. The tubes were then cut into the longest straight lengths which could be obtained (3 - 6mm.). Usually only one or two such lengths could be obtained from any one tube. The cuts were made either with a junior hacksaw fitted with a fine blade or with a small abrasive cutting disc mounted on a Dremel 396-3960 modelmaker's tool. Cutting was continued until the substratum was penetrated to a depth of around 1mm. The Dremel proved to be the better tool for the purpose as it gave a smooth, clean cut, seldom dislodged the piece of tube from the test surface and penetration of the substratum was limited to the immediate area of the tube. The perimeter of the base of the tube section was then marked as in method 1. A sewing needle or small

length of stainless steel wire with a diameter which matched the inner tube diameter as closely as possible was then passed through the section of tube and over a piece of terylene whipping twine, which had been looped at both ends. These loops were then inserted into the grooves which had been cut into the substratum (Fig. 2). Without such grooves in the substratum it was difficult to pass the loops of terylene whipping twine around a well-fitting needle without dislodging the tube from the substratum.

Two methods of attaching *Laminaria digitata* to experimental surfaces were tried - propagation from zoospores and the transplantation of young sporophytes.

Propagation was achieved by removing sections of blade containing sori, wiping them clean to remove epifauna and flora and cutting them into 10mm. squares. These squares were then placed into a beaker containing 100ml. of fine filtered and irradiated seawater (FFSW). After 1 - 4 hours motile zoospores were released and could be detected by a slight cloudiness of the water. These zoospores were pipetted off from mid-water, to avoid picking up diatoms, and added to beakers containing test substrata and a culture medium consisting of 1ml. l^{-1} Conway's medium in autoclaved FFSW. Germanium dioxide was added to this culture medium at the rate of 6µg. l^{-1} in order to reduce the growth of diatoms. The beakers containing the zoospores were then kept at ambient seawater temperature by placing in a tank of running seawater. Irradiation at 2.2×10^{15} Q cm^{-2} sec^{-1} was provided by daylight fluorescent tubes suspended above the tank.

Male and female gametophytes were well developed by *circa* 18 days and young sporophytes were formed at *circa* 30 days. At *circa* 40 days the young sporophytes had clearly defined blades and small stipes and were anchored to the substratum by rhizoids (Fig. 3). At this stage the

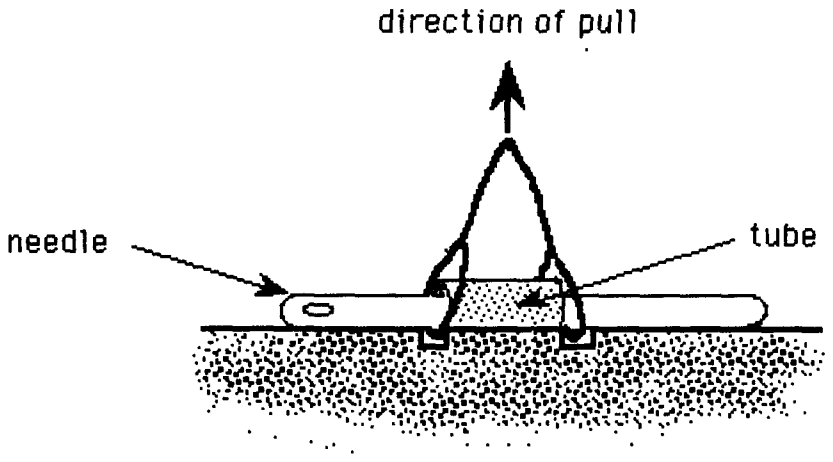


Fig. 2: The method used to attach small pieces of *Pomatoceros lamarckii* tube to the force gauge. Note the grooves cut into the substratum to accommodate the terylene cord.

sporophytes were thinned to leave four to six healthy plants per 20 x 50 x 3.5mm. slate, then transferred to running seawater in natural light and left to grow until required. After thirty days in running seawater a small, round holdfast developed which quickly divided to produce primary haptera.

This method of attaching *Laminaria digitata* to experimental surfaces, although useful for observing the rhizoidal attachment of young sporophytes and the development of haptera, was very slow and not really suited to obtaining mature plants on experimental surfaces. Transplantation of young sporophytes proved to be more advantageous for this purpose.

Tovey & Moss (1978) attached *L. digitata* sporophytes to experimental surfaces by cutting from young plants sections of stipe with a whorl of young haptera which had not yet reached the substratum. The holdfast below these young haptera was also removed and the pieces of stipe

Figure 3.

Light micrograph of young *Laminaria digitata* sporophytes *circa* 40 days old. Scale bar...300 μm.

Inset: Rhizoids at higher magnification. At the base of the stipe the small disc from which haptera will grow is just beginning to form. Scale bar...110 μm. b...blade. st...stipe. r...rhizoids.

Figure 7.

Scanning electron micrograph showing the formation of rhizoids by the elongation of meristoderm cells (m). Those cells in the foreground can be seen to be starting to elongate, the cells in the background have become more elongated and some rhizoids (r) have formed. Scale bar...10 μm.

Figure 8.

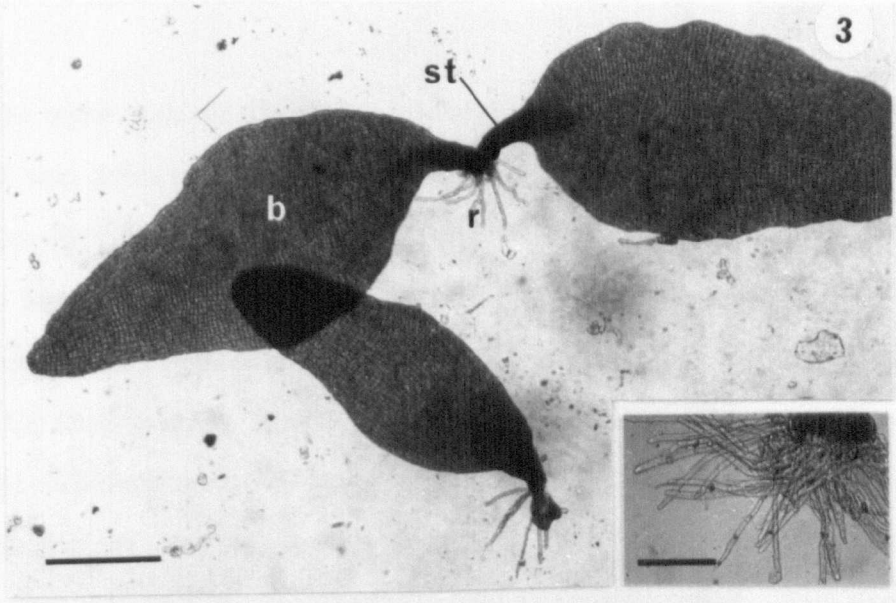
Scanning electron micrograph of plankton net which is densely interwoven with *Laminaria digitata* rhizoids. Scale bar...120 μm.

Figure 9.

Scanning electron micrograph of rhizoids spreading along a substratum. Eventually the posterior areas coalesce to form columnar pseudoparenchyma (pp). Scale bar...60 μm.

Figure 10.

Scanning electron micrograph of densely packed rhizoids forming a perfect cast of the plankton net from which they have been removed. Scale bar...80 μm.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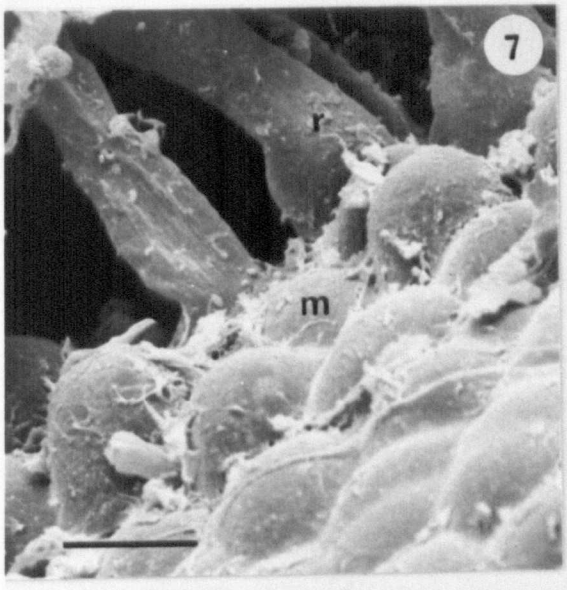
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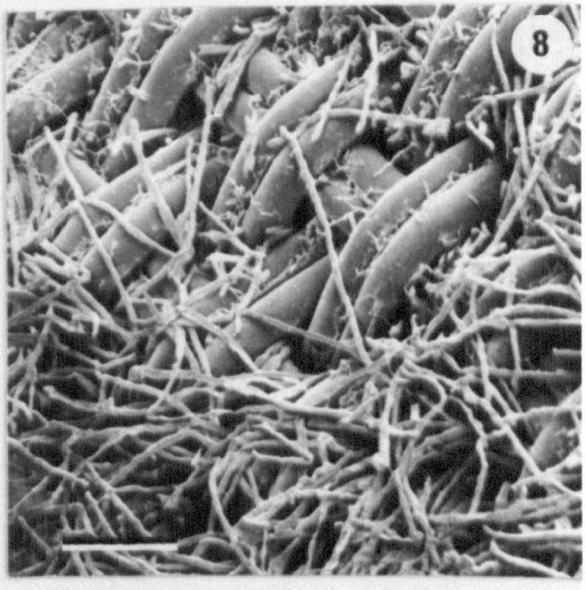
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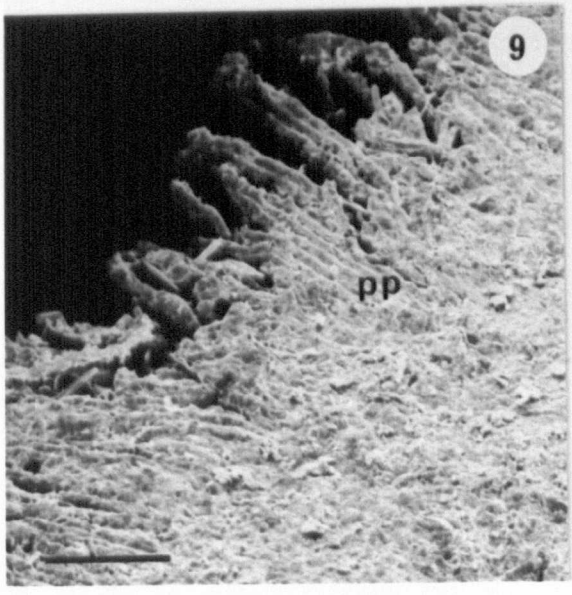
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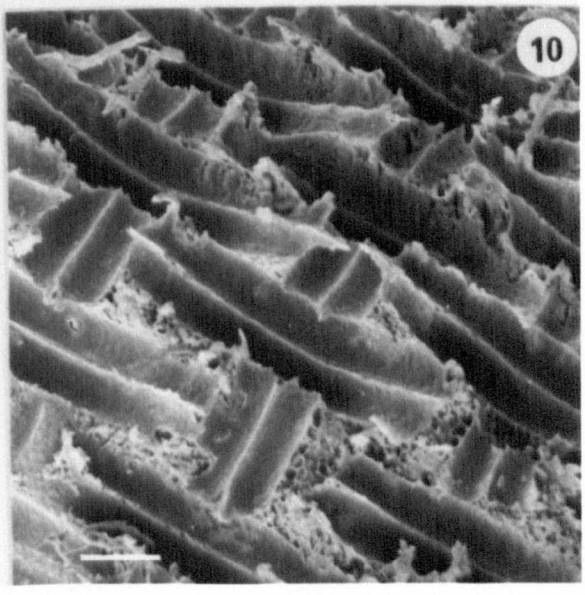
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with haptera were then attached to a variety of substrata. Attachment to the substratum was achieved in 4-12 weeks. This method was repeated in the present study but proved unsuccessful, as the pieces of stipe always rotted before any sign of attachment. Therefore whole, young sporophytes, 50 - 75mm. long, were obtained from the Menai Strait and attached to experimental substrata by tying the haptera down onto the surface of the substratum with terylene whipping thread. The prepared surfaces were then maintained in running seawater and natural light. This method of transplantation was extremely successful and relatively quick. The existing haptera in contact with the slate did not attach but new haptera grew and, after contact with the substratum was made, attached in *circa* 21 days. The plants continued to grow vigorously and were maintained in running seawater until required. Some plants remained healthy when maintained in this way for more than a year, attaining a length of 450mm. with a maximum blade width of 150mm.

This transplantation technique was also used to grow *Laminaria digitata* on a variety of substrata in order to investigate the way in which it adheres to a substratum.

The shape, fragility and mucous coating of *L. digitata* stipes and haptera made the attachment of a loop with which to connect the stipe or hapteron to the force gauge something of a problem. The solution was provided by a knot used in mountaineering, the Prusik knot (Fig. 4).

This knot can be slid along the stipe or hapteron when not in tension but when the free loop is loaded the knot tightens and grips the stipe with increasing strength as the load is increased. Used alone the knot could sever a small stipe or hapteron at high load. Such mechanical damage was prevented by wrapping a piece of fine grade glass paper, rough surface inwards, around the stipe or hapteron to be pulled. A Prusik knot was then

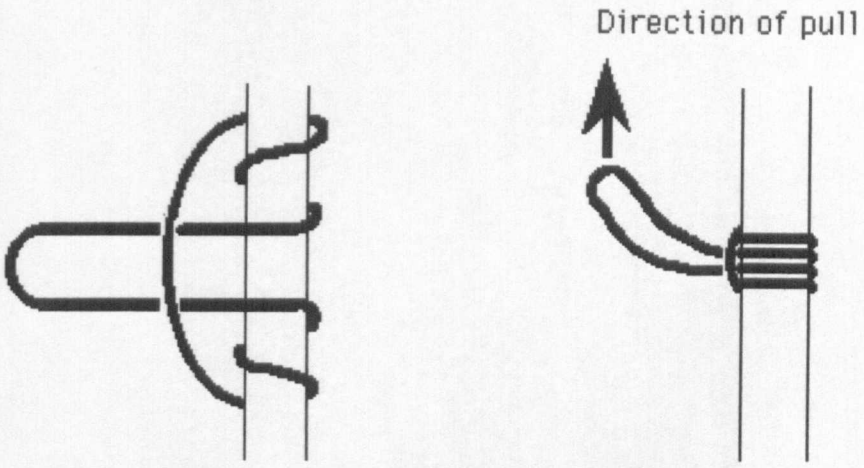


Fig. 4: The method of tying the Prusik knot, a self-tightening friction hitch used to attach *Laminaria digitata* stipes and haptera to the force gauge.

tied around the glass paper using a loop of terylene whipping twine. In addition to preventing damage, the glass paper also provided an increased grip on the mucous coated surface.

Another factor which has to be taken into account when pulling *Laminaria digitata* is the direction in which the pull needs to be applied to give an accurate estimate of the tenacity of the holdfast. The holdfast as a whole tends to grow in the direction which will best resist the forces to which it is subjected by water flow. Individual haptera echo this trend and any attempt to pull a stipe or hapteron at right-angles to the substratum causes the plant to peel easily off the substratum thus giving a deceptively low estimate of its tenacity. Fig. 5 shows the method which was devised to overcome this problem. The loop of terylene whipping twine was made sufficiently long to be passed under a round steel bar which was clamped to the base of the force gauge at such a height that the pull would be transferred to the hapteron along its direction of growth.

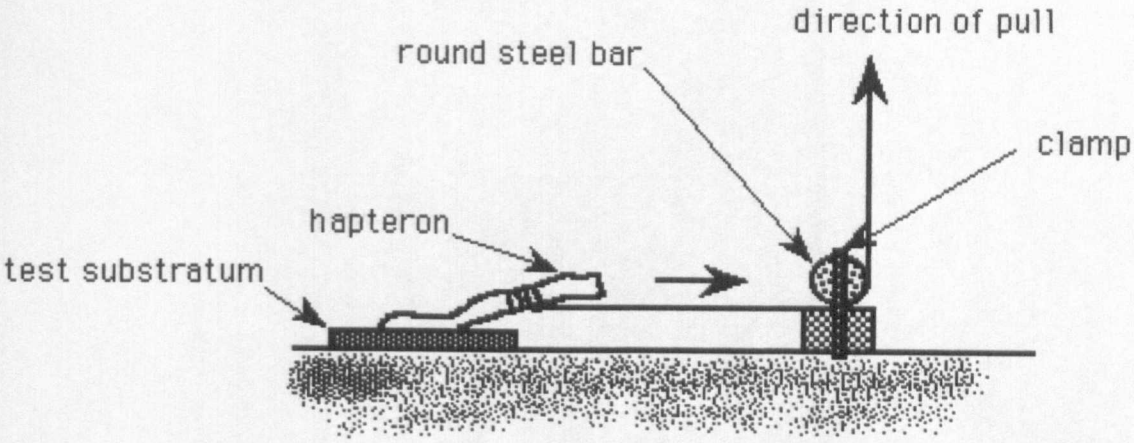


Fig. 5: Rig for pulling *Laminaria digitata* haptera in the direction of growth.

Only two pulls were made using complete holdfasts. These pulls gave a low result due to delamination and are not included in the results. Subsequent pulls were made on individual haptera using the technique described above. Prior to pulling, the outline of the attachment area of the hapteron was marked onto the substratum as described for *Pomatoceros lamarckii* to allow measurements of area to be made.

Measurement of tenacity

Pieces of prepared *P. lamarckii* tube and *Laminaria digitata* haptera were pulled normal to the substratum to which they were attached using an Antana AFG3 digital electronic force gauge with an operating range of 0 - 25kg. This force gauge was mounted on a custom-built stand which enabled the specimen to be positioned centrally below the attachment hook of the force gauge and to be securely held in place by clamps (Fig. 6). The stand also allowed coarse and fine vertical movement of the gauge so that it was possible to achieve a smooth, continuous pull. The force gauge was

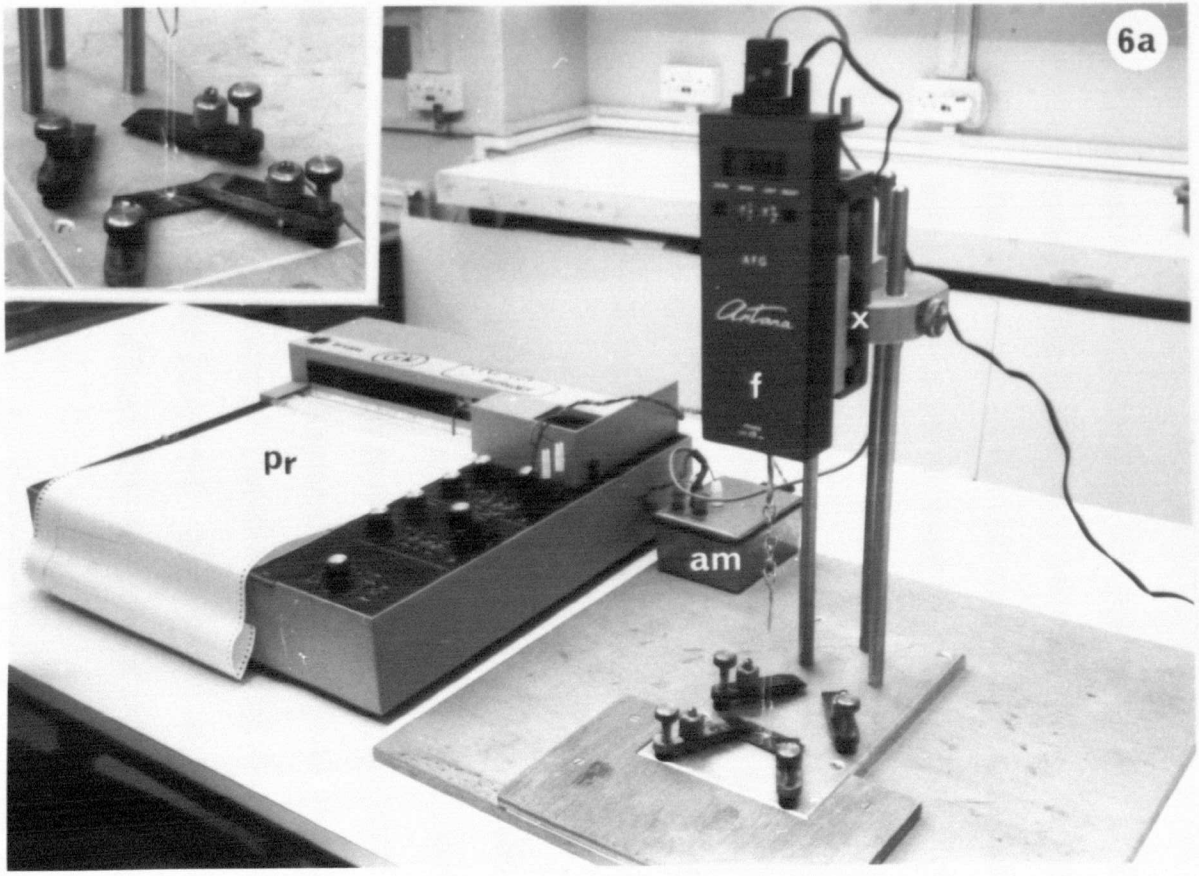
Figure 6a.

The Antara AFG3 force gauge (f) attached to a custom-built stand (x) and connected via an amplifier (am) to the Bryans 28000 pen recorder (pr).

Inset: Detail of the specimen securing clamps and the arrangement for pulling a *Pomatoceros lamarckii* tube.

Figure 6b.

Side view of the custom-built stand (x) and force gauge (f). Coarse height adjustment is made by sliding the stand on its supporting rods and locking it into position with the nuts (l). Fine adjustment/pulling is achieved by turning the fine threaded rod (t) using the knurled wheel (w).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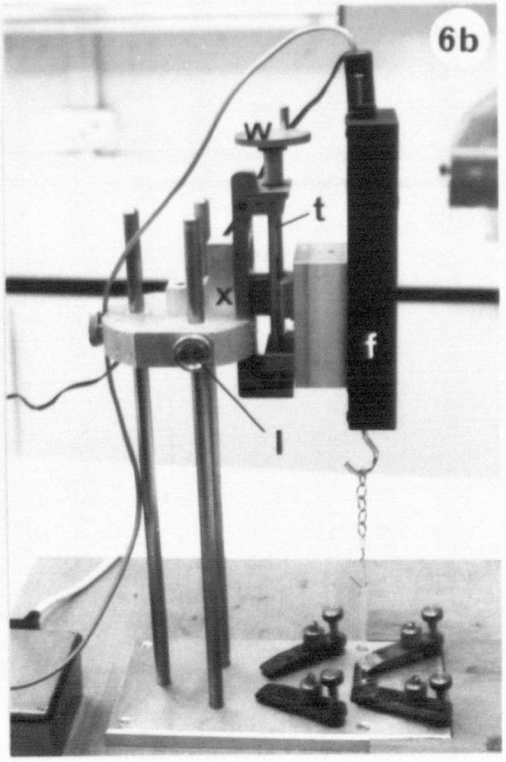
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connected to a Bryans 28000 pen recorder in order to obtain a permanent record of each pull. Standard metric weights between 50gm. and 3kg. were hung from the force gauge as calibration weights before and after experimental force measurements. Initially, test pulls were made using pieces of monofilament nylon fishing line of known breaking strain and it was discovered that the force gauge always registered a lower (digital) readout than that on the pen recorder. Further testing revealed that the force gauge had a slight time lag before the digital readout appeared. A short, sharp pull on the attachment hook gave no reading on the force gauge but was instantaneously registered by the pen recorder. This time lag proved to be the reason for the discrepancy between gauge readout and pen recorder trace. When the break in the test material occurred the pen recorder recorded the actual force at the moment of breaking but the force gauge only recorded the force which was being exerted a fraction of a second before the moment of breaking. It was therefore decided that the pen recorder trace provided a more accurate (and permanent) record of the force at the moment of breaking. Readings of both the force gauge and the pen recorder were recorded but only those of the pen recorder were used in the tenacity calculations.

The results of four early pulls were discounted as these pulls were made with the force gauge alone and were considered to be inaccurate. Materials to be pulled were not allowed to dry out and all pulls used in the results were made with the tube or hapteron and substratum wet, as this condition approximated to the normal situation of the tube or hapteron. Some dry pulls of *Pomatoceros lamarckii* tubes were made and it was found that the forces recorded differed little from those recorded from wet pulls.

Following force measurement, the shape of the base of the tube or hapteron was drawn onto graph paper, using a *camera lucida* fitted to a

Wild stereomicroscope and the total attachment area in square mm. was calculated from this drawing. The area which had been pulled (if part of the base remained attached to the substratum) and the area of any surface which was removed during the pull were also drawn in and measured.

Forces were measured in Newtons (N) and tenacity (force per unit area) was calculated using the formula:

$$T = \frac{F}{A} \times 10^6$$

Where T = Tenacity (N m^{-2})

F = Force (N)

A = Area of tube/hapteron removed by pull (mm^2)

An adjusted figure, allowing for the effect of the area of any substratum removed, was arrived at by subtracting the area of substratum removed from the area of tube or hapteron removed by the pull and substituting the result for value A in the above equation. In effect the calculation was made on the area of cleanly pulled tube or hapteron. When the area of pulled substratum greatly exceeded that of cleanly pulled tube or hapteron this method gave a highly inflated adjusted figure (see Discussion). Such cases (six in all) were not included in the results.

RESULTS

The overall mean tenacity figures for *Pomatoceros lamarckii* tube pulled from rough and smooth slate and stones were reasonably consistent. Force measurements carried out on tubes attached to the rougher substrata resulted in slightly higher figures than the smooth (Table 1). The hardwood figure, however, was an order of magnitude less than the figures

for slate and stone and can probably be discounted as it represents a single pull in which the animal could have been poorly attached or the fibres of the wood weak or loose. Several pulls on this type of substratum would be required to obtain a more realistic figure.

Table 1: Mean tenacities (± SD) of *Pomatoceros lamarckii* tube on a variety of substrata, including adjusted tenacity figures when areas of substratum were removed by the pull.

Substratum	Mean Tenacity ($N\ m^{-2} \times 10^5$)	Adjusted Tenacity ($N\ m^{-2} \times 10^5$)	Sample size
Smooth slate.	15.2 ± 9.8	16.5 ± 10.0	15
Rough slate	17.2 ± 5.6	17.9 ± 4.8	4
Stones (mica schist)	17.3 ± 10.3	18.3 ± 9.0	4
Hardwood	0.5	0.9	1

If the tenacity figures for clean pulls are considered in isolation, tubes which were attached to smooth slate would appear to have a higher tenacity than those attached to the rougher substrata (Table 2). However, sample sizes for clean pulls are small which probably explains this anomaly.

Only one successful whole tube pull (on a smooth slate substratum) was made. This pull resulted in an adjusted tenacity figure of 16.4×10^5 N m^{-2} which compares favourably with the figures obtained from part tubes on the same substratum.

Table 2: Mean tenacities (± SD) of *Pomatoceros lamarckii* tube on a variety of substrata, based on clean pulls only.

Substratum	Mean Tenacity ($N\ m^{-2} \times 10^5$)	Sample size
Smooth slate.	24.75 ± 6.7	6
Rough slate	21.9 ± 2.1	2
Stones (mica schist)	22.2 ± 8.3	2

Young *Laminaria digitata* sporophytes remained attached to the female gametophyte during early cell division. As growth continued rhizoids were extended which secured the sporophytes to the substratum. These rhizoids stained heavily with Alcian Blue indicating the presence of weakly sulphated acid mucins forming a mucous coat around the rhizoids. As growth continued a round, primary holdfast was formed which, in turn, divided into primary haptera as described by Yendo (1911). Further rings of secondary haptera were formed above the primary haptera.

Contact of haptera with a substratum resulted in the formation of rhizoids from the meristoderm cells (Fig. 7). It is thought that contact with the substratum is the trigger for rhizoid production (Tovey & Moss, 1978). These rhizoids elongated and spread along the surface of the substratum at the outer edges of the haptera. Any gap or crevice in the substratum was penetrated by the rhizoids (Fig. 8). As the rhizoids continued to extend across the substratum their proximal ends became bonded together, in a matrix composed of their surrounding mucus, to form a solid, columnar pseudoparenchyma which moulded precisely to the contours of the substratum (Fig. 9). Eventually the haptera conformed exactly to the shape

of the substratum. This conformity is well demonstrated by growing *Laminaria digitata* on plankton net (Fig. 10).

Young *L. digitata* sporophytes can often be found attached to other plants of the same or different species. SEM examination of the junction between the haptera of *L. digitata* and its host plant showed this junction to be extremely intimate. The cells of both plants reduced in size until they finally merged into a junction which appeared to be filled with a fibrous material and/or mucus (Fig. 11).

The above observations on the way in which the haptera and rhizoids of *L. digitata* grow and conform to substrata support the observations of Tovey & Moss (1978) with the exception of one detail. Tovey & Moss stated that the rhizoidal cells continued to elongate until they met a solid object when growth ceased. This cessation of growth was not always the case in the present study. Whether a rhizoid ceased to elongate or not appeared to depend upon the shape and size of the solid object and the angle at which the object was encountered. Fig. 12 shows a rhizoid which encountered a piece of foam plastic, became flattened in section and carried on elongating across the surface of the plastic.

Pulling results for *L. digitata* were treated in the same way as for those of *Pomatoceros lamarckii* with tenacities of all pulls adjusted to allow for areas of substratum detached during pulling (Table 3) and the tenacities calculated for clean pulls given separately (Table 4).

The standard deviation on these pulls is not so great as on the *Pomatoceros lamarckii* pulls which probably results from the fact that the adhesion of the *L. digitata* haptera was in excess of their cohesive strength. Thus the haptera always broke (usually at the narrowest part) before really high tenacity figures were reached, leaving the base of the

Table 3: Mean tenacities (± SD) of *Laminaria digitata* haptera on smooth and rough slate with adjusted tenacity figures when areas of substratum were removed by the pull.

Substratum	Mean Tenacity ($N\ m^{-2} \times 10^5$)	Adjusted Tenacity ($N\ m^{-2} \times 10^5$)	Sample size
Smooth slate.	>2.68 ± 1.8	>2.89 ± 1.6	9
Rough slate	>4.14 ± 1.9	>4.21 ± 1.9	3

haptera still firmly attached to the substratum. There was little difference between the results for all pulls (Table 3) and those for clean pulls only (Table 4).

Table 4: Mean tenacities (± SD) of *Laminaria digitata* haptera on smooth and rough slate substrata, based on clean pulls only.

Substratum	Mean Tenacity ($N\ m^{-2} \times 10^5$)	Sample size
Smooth slate.	>3.33 ± 1.9	6
Rough slate	>4.22 ± 2.7	2

The preliminary tenacity figures obtained by Young (unpublished, quoted in Walker (1987)) for both *Pomatoceros triqueter* (>20 x 10^5 N m^2) and *Laminaria digitata* (5.3 x 10^5 N m^2) correspond quite closely to those obtained in the present study.

DISCUSSION

Pulls in which pieces of the substratum were detached along with the *Pomatoceros lamarckii* tube were difficult to interpret (*Laminaria digitata* pulls did not remove substantial areas of substratum). In some cases a considerable amount of the substratum was removed. In this case the tenacity figure which was obtained largely reflected the cohesive strength of the substratum and it could be said with some certainty that the tenacity of the tube was therefore somewhat greater than this figure. In other cases the amount of substratum removed was minimal and consisted of small, scattered areas and/or a very thin layer. Again the true tenacity of the tube would have been higher but in this instance the amount of substratum which was removed probably had little effect on the result and the method used to arrive at an adjusted tenacity probably resulted in a reasonably accurate estimate of tube tenacity.

The most accurate results to be obtained, however, must be those in which the pull was clean with no substratum removed. Unfortunately the sample size of these results was low for both *P. lamarckii* and *L. digitata*, particularly for the rough slate (2 pulls) and the mica schist stones (2 pulls) which probably leads to a distorted view of the tenacity relative to surface contour. If, as will be argued, the adhesion of *P. lamarckii* is purely mechanical one would expect the relatively smooth surface to provide the lowest tenacity figures but the reverse is the case with the figures obtained from the clean pulls. The overall figures (Table 1) do support the argument for mechanical adhesion with the rougher surfaces producing the highest tenacity figures. These discrepancies can probably be explained by the small sample size of the clean pulls. Ideally many more pulls should be made which would increase the chances of obtaining a large sample of clean

pulls and, in turn, result in a more accurate assessment of the tenacities of both *Pomatoceros lamarckii* and *Laminaria digitata.*

Interestingly the lowest tenacity figures obtained for *P. lamarckii* (excluding those from tubes on wood) all came from pieces of old tube, the lowest figure recorded being 3.9×10^5 N m^{-2}. This result could be due to chance as it is only based on four pulls of old tube but it could also be due to an age related phenomenon.

Examination under both light microscope and SEM of *P. lamarckii* tubes attached to substrata revealed no trace of a specific substance, other than the mucus which makes up the tube matrix, which could be classed as an adhesive. Observation showed that this mucus often extended slightly beyond the calcified area of the tube at the external tube - substratum interface and normally formed a sheet across the base of the tube interior. Without calcification mucus possesses little inherent tensile strength and cannot account for the measured tenacity of the tube. Suction may also be ruled out as a means of adhesion as the strength of adhesion is well above that which would result from atmospheric pressure (atmospheric pressure is approximately 1×10^5 N m^{-2}).

During the course of this study it has frequently been observed that a tube formed on a smooth, glass sheet can easily be pushed sideways. Occasionally it could be seen that small pieces of mucus still adhered to the glass where the animal was originally situated, indicating that this mucus offered the main resistance to the shearing force being applied. Any attempt to slide a *P. lamarckii* tube sideways across a slightly rough surface, such as a piece of smooth slate, required the application of much higher shear forces and resulted in major damage to the tube or detachment of the tube plus a section of the substratum. In the absence of a strong adhesive the only explanation for this resistance to high shear forces is that adhesion is obtained by mechanical interlocking of the calcified tube within

the irregularities of the substratum. For this mechanical interlocking to be really effective it would be necessary for the tube to be able to conform to every microscopic irregularity.

SEM observations have confirmed that the tube is able to conform exactly to the surface contour of a substratum. In Fig. 13 it can be seen that small rods of calcium carbonate are formed around any irregularity, filling all gaps. Figs. 14a and 14b illustrate just how fluid and fine the initial mucus/calcium carbonate mix must be when released. Fig. 14a shows a row of bordered pits in the xylem of a piece of hardwood, one of which is covered by *Pomatoceros lamarckii* tube material. Fig. 14b shows an inverse image of the bordered xylem pits formed in the base of a *P. lamarckii* tube. This tube was attached to the piece of hardwood shown in Fig.14a. This degree of conformity to the substratum creates a very strong mechanical bond which is reflected in the tenacity figures.

The pulling data obtained for *Laminaria digitata* showed it to have a lower tenacity than that of *P. lamarckii* but this tenacity was still greater than that which would result from atmospheric pressure alone. Microscopy has revealed that *L. digitata* is able to achieve an extremely intimate mechanical bonding with every contour and crevice of a substratum and it is largely this mechanical bonding between holdfast and substratum which holds the plant firmly in high energy environments. The literature offers reasonable evidence to suggest that this bonding is further enhanced and strengthened by the mucous coating of the rhizoids, which appears to be a common feature of many of the more advanced marine algae (Moss, 1973; Tovey & Moss, 1978; Hardy & Moss, 1979; Fletcher, 1980). It may therefore seem surprising that the mean tenacity recorded was less than that of *P. lamarckii*. There are three reasons why this disparity occurs. The first of these reasons is that the experimental substrata offered little in the way of surface contour which could be exploited by the ability of the

Figure 11.

Scanning electron micrograph of a section through the junction between a *Laminaria digitata* hapteron (ld) and the stipe of a *Fucus* species alga (fs). the cells of both plants become progressively smaller towards the junction, which appears to be filled with a fibrous material. Scale bar...50 μm.

Figure 12.

Scanning electron micrograph of a rhizoid (r) contacting a piece of foam rubber. The rhizoid (growing from the right) has hit the foam rubber and carried on elongating across it. Scale bar...5 μm.

Figure 13.

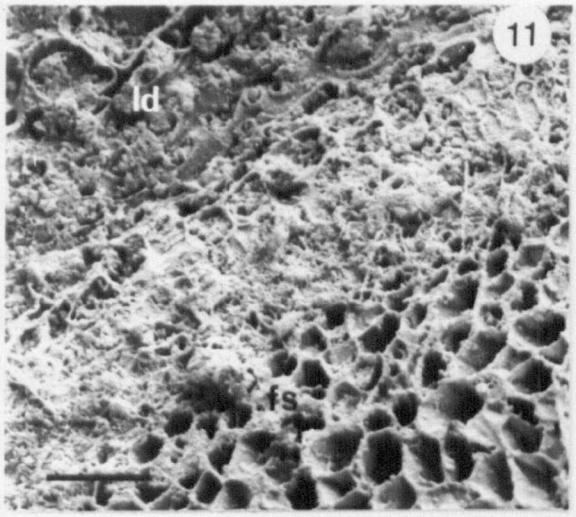
Scanning electron micrograph showing how the calcium carbonate-/mucus matrix of a *Pomatoceros lamarckii* tube surrounds, and conforms very closely to, slate particles (sp). Scale bar...5 μm.

Figure 14a.

Scanning electron micrograph of bordered pits (bp) in the xylem of a piece of wood. *Pomatoceros lamarckii* tube material has commenced to flow into one of the pits (arrow). Scale bar...25 μm.

Figure 14b.

Scanning electron micrograph of calcareous, *Pomatoceros lamarckii* tube matrix moulded into the shape of the bordered pits with which it has been in contact. Scale bar...25 μm.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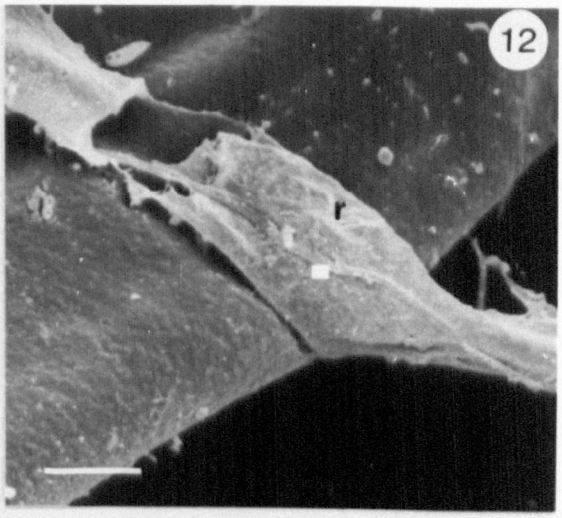
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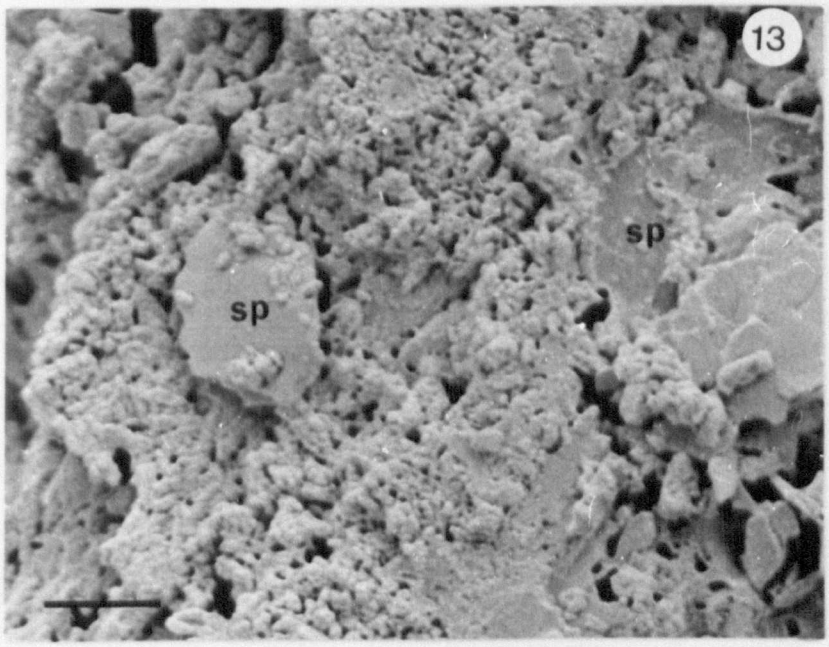
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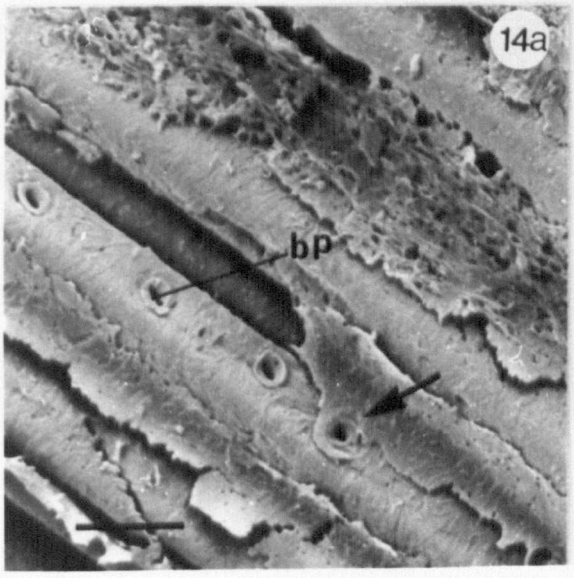
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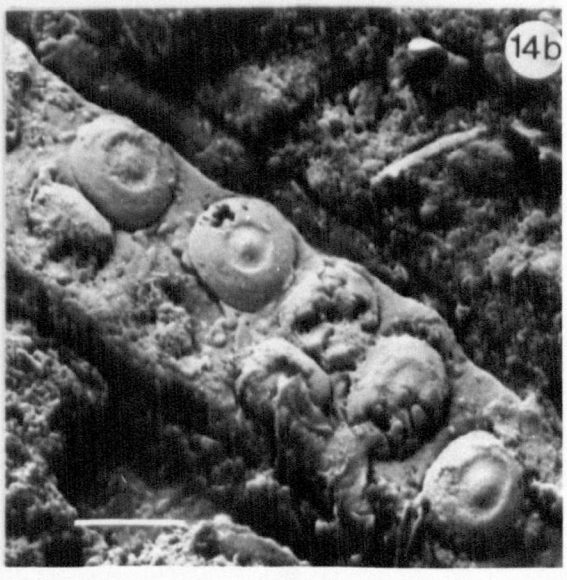
14b

rhizoids to infiltrate every crevice in a substratum. Secondly, the angle at which the haptera were pulled was critical. A pull which was made slightly out of line with the direction of growth of a hapteron always resulted in some degree of delamination occurring immediately prior to the detachment of the hapteron. This delamination resulted in a lower tenacity figure than might otherwise have been achieved. Finally, and probably most significantly, when a pull was well set up to provide an optimum result the hapteron itself always failed below 6.5×10^5 N m^{-2} leaving its base still firmly attached to the substratum. It can therefore be stated with some certainty that, in situations where the surface contour of the substratum offers good opportunities for mechanical attachment, the adhesion of the *Laminaria digitata* holdfast will be in excess of 6.5×10^5 N m^{-2} and will normally exceed the tensile strength of the individual haptera and stipe. Confirmation of this holdfast strength can be found in kelp beds which are exposed to strong currents and subject to storm damage where holdfasts with short stubs of broken stipe are not uncommon. Of course the flexibility of the *L. digitata* stipe allows the plant to reduce considerably the forces upon the holdfast and upon the plant as a whole and it can reasonably be assumed that these breakages normally occur only among older, senescent plants or under extreme conditions.

Whole *L. digitata* sporophytes are occasionally to be found on beaches following a storm. Examination of the holdfasts of these plants normally reveals that the holdfast has overgrown other encrusting organisms, e.g. barnacles, which have subsequently died and decayed, thus creating a weak interface between holdfast and substratum. It is this intermediate layer which has pulled away from the substratum causing the sporophyte to become detached.

Biological adhesives can be divided into those which are temporary and those which are permanent (Crisp, 1972). Among animals which

employ temporary adhesives are those which have to be able to move at times but also require a strong attachment to the substratum at others. *Patella* species, for example, have to move to forage but must be able to achieve a rapid and strong attachment when threatened by predators or by dislodgement due to wave action. Adult barnacle adhesive is considered to be permanent but adults are able to move sideways in situations of overcrowding. This ability to move sideways is thought to be due to barnacle cement acting as a viscous fluid (Crisp, 1972) which results in the cement having a low resistance to shear forces (Walker, 1987). Table 5 gives the tenacities recorded for several organisms which employ temporary or permanent adhesives.

Of the three species of limpet for which tenacity figures have been given, *Patella vulgata* is the common species in waters around Britain, *P. granularis* is a tropical species from a sheltered environment and *P. cochlear* is a tropical species from an environment where there is strong wave action. It can be seen that *P. cochlear* has, as one might expect from its environment, the highest tenacity of the three species. *Balanus balanoides* has a markedly higher tenacity than those organisms employing a temporary adhesive.

The present results show that both *Pomatoceros lamarckii* and *Laminaria digitata* adhere to the substratum by mechanical adhesion based on their respective capabilities to conform exactly to the contour of the substratum. A comparison of these species and those in Table 5 shows that *L. digitata* has a tenacity which is similar to those species which employ a temporary adhesive. *P. lamarckii* has a considerably higher tenacity than that of *Balanus balanoides*, the only representative in Table 5 of organisms employing a permanent adhesive.

Table 5: Mean tenacities (± SD) of a variety of marine organisms. (t) = temporary adhesive. (p) = permanent adhesive.

Species	Tenacity ($N\ m^{-2} \times 10^5$)	Reference
Barnacles (p):		
Balanus balanoides	9.3 ± 4.9	Yule & Walker (1984)
Limpets (t):		
Patella vulgata	2.3 ± 0.2	Grenon & Walker (1981)
P. granularis	3.3 ± 0.8	Branch & Marsh (1978)
P. cochlear	5.2 ± 0.9	Branch & Marsh (1978)
Anemones (t):		
Actinia equina	4.6 ± 1.26	Young *et. al.* (1988)

Chapter 8

General Discussion

The underlying aim of any living organism is to perpetuate the species and, in so doing, to pass on its own genetic material to future generations. In order to achieve this aim the organism has to be well adapted to an ecological niche which provides adequate food, shelter, few predators and the presence of others of its own species. The selection of a suitable habitat is of particular importance to a sessile organism such as a sedentary polychaete worm, as once its larva has settled and metamorphosed into the sessile adult form there is a total commitment to that site for life. Inability to select a habitat with the necessary requirements for survival and propagation of the species may result in the organism failing to reach reproductive age or being unable to pass on its genetic material to others of its species.

Meadows & Campbell (1972) defined habitat selection as the relationship between behaviour and environment and stated that this relationship largely determines the local distribution of a species. They stated that animals find a suitable habitat by a process of choice, assessing and responding to various cues from the environment. While this general statement is undoubtedly true, one has to consider what is meant by 'choice' in the context of larval biology. Choice implies a conscious decision-making process but simple animals, lacking a well-developed nervous

system, are not likely to have decision-making capabilities and their actions are simply a response to particular stimuli. Such animals may thus appear to choose, for example, between different substrata upon which to settle but in reality they investigate a number of substrata until one substratum triggers a settlement response. Chia (1978) declared that gregarious and sedentary species are more dependent on environmental stimuli for induction of settlement and metamorphosis than non-gregarious and mobile species. Natural selection ensures that those animals which make the correct responses to these stimuli are the most likely to survive. Observations of the behaviour of marine invertebrate larvae should thus be capable of interpretation in terms of benefit to the species, although it is often difficult to arrive at such a logical interpretation on the basis of available evidence.

Intertidal species inhabit an environment which is characterised by widely fluctuating parameters. For example, a sedentary polychaete worm in a tube attached to the upper surface of a boulder will, in the temperate zone, on a hot summer day, experience a change from 100% humidity and an ambient temperature of around 17°C to very low humidity and an ambient temperature of around 28°C and back again within a single tidal cycle. In order to survive in such an environment a species has to be able to withstand exposure to air, desiccation, extremes of temperature, fluctuations in pressure and, on occasion, exposure to low salinities or actual immersion in freshwater. *Pomatoceros lamarckii* is found mainly in the mid-littoral zone while *P. triqueter* is largely confined to the sub-littoral zone. Both species are able to survive long periods of emersion due to the ability to seal off the openings of their calcareous tubes with tightly fitting opercula and it is likely that the limiting factors which govern the

time which they can spend out of water are oxygen supply and ambient temperature. So far as can be ascertained, no work has been carried out on these aspects of the biology of *Pomatoceros* species.

A *Pomatoceros* species metatrochophore larva which is about to settle must, therefore, be genetically programmed to locate and explore, a hard substratum in or below the mid-littoral zone. Ideally this substratum will be situated in water with sufficient movement to keep the animal free from sediment and to carry a steady flow of food particles. Finally, the larva is stimulated to settle sufficiently close to conspecifics so that efficient exchange of gametes will take place later in the life cycle.

The present study has shown that (in the laboratory) *Pomatoceros lamarckii* larvae are, initially geonegative and are to be found at the water surface shortly after becoming mobile. They are, overall, negatively phototaxic throughout life but also display brief periods of positively phototaxic behaviour. Meadows & Campbell (1972) state "....polychaetes are photonegative under water", but it is unclear whether this generalisation is meant to embrace both sedentary and errant polychaetes and whether it also includes larval forms. Workers concentrating on the responses to directional light of individual species of polychaetes have found a variety of responses (Thorson, 1964; Young & Chia, 1982; Marsden, 1984, 1986, 1988, 1990) which vary according to the requirements of the species.

It is not entirely clear what advantage *P. lamarckii* gains by remaining photonegative throughout its larval life. The answer probably lies in the period of larval dispersal which allows the possibilities of wider genetic exchange, potential to colonise distant geographical areas and the ability to occupy an 'impermanent' habitat (Crisp, 1974). For larval dispersal to be really effective larvae have to be situated in the most favourable current.

Friction at the sea-bed ensures that any current will be weaker close to the substratum. The initial period of geonegativity, when the larvae first become motile, will cause the trochophore larvae to ascend towards the surface and thus into more favourable water flow and at the same time will put them beyond the reach of benthic predators. However, increasing photonegativity would appear to negate the tendency to rise towards the surface and would cause the animal to descend in the water column. The key to this anomaly is possibly to be found in the short period of positive phototaxis which precedes negative phototaxis. By alternating periods of positive and negative phototaxis the larvae would be able to maintain a favourable level in the water column, neither sinking too low nor rising too close to the surface. It is feasible that larvae which remain relatively low in the water column suffer less predation than those in the bulk of the plankton. There are also inherent dangers for naked larvae in becoming trapped in the surface film and being torn apart by surface tension. Thus the disadvantages of slower transport and growth could be offset by the survival of more larvae which are able to settle and metamorphose. As the periods of photonegativity become longer and the negative photoresponse becomes stronger, the tendency would be for the larvae to slowly descend in the water column, finishing in close proximity to a substratum as they approach readiness to settle and metamorphose.

Alternatively, it may well be that the laboratory results do not give an entirely true indication of what actually takes place in the field. Temperature, pressure, salinity, light direction and light intensity are all known to affect the photoresponse of marine invertebrate larvae (Ryland, 1960; Thorson, 1964; Crisp & Ritz, 1973; Forward, 1974). Sulkin (1990) throws doubt on those methods of testing for photoresponse which employ

a highly directional light source and/or horizontal light, arguing that these conditions are not normally found in nature. He also states: "Each organism may have a characteristic stimulus threshold (time or intensity) above which it changes from indifference to either positive or negative (or from positive to negative)".

It is relatively easy to ensure that a series of experiments is carried out with the same regime for temperature, pressure, salinity, light intensity and direction. Variation of any one of these parameters does not present any great difficulties in the laboratory, but it is a great deal more difficult to permutate the combinations which are possible when one starts to vary all of these parameters. Attempts to ascertain which of these combinations are applicable in nature would also present complex problems. Future research, however, should attempt to take the above factors into account if we are to arrive at a more accurate assessment of the photoresponse of marine invertebrate larvae.

As metatrochophore *Pomatoceros lamarckii* larvae approach settlement they become longer and more worm-like in appearance. The time taken to arrive at this stage can vary quite considerably from one batch of larvae to another and also within a particular batch. The majority of larvae in a batch can become competent, i.e. ready to settle, as early as day eight from the time the larvae become free-swimming or as late as day sixteen. Within any batch one will also find larvae which are much smaller than the mean size at any given time. For example, the mean larval size at day twelve was found to be 315 x 175μm. but perfectly formed larvae of 200 x 130μm. were also to be found. This variation in size could perhaps be accounted for by a shortage of food, but as food was always plentiful in

laboratory cultures this explanation seems unlikely. The more probable explanation is that this size variation is a result of genetic variability and is basically a survival strategy. Larvae at various stages of development within one batch could ensure that, in the event of the majority of larvae reaching competence in an unfavourable location, some larvae would survive to carry on the dispersal and possibly reach a suitable settlement site by the time competence was attained.

Segrove (1941) recorded that fully developed, *Pomatoceros triqueter* metatrochophore larvae could remain free-swimming for up to eight weeks but gave no indication that such larvae were still capable of settlement and metamorphosis. There is no reason to suspect that such larvae, if still healthy and active, would not be able to settle given a suitable stimulus. Some specialised pelagic larvae are capable of settlement even after a period of six months in the plankton (Scheltema, 1986). The fact that Segrove (1941) was able to keep *P. triqueter* larvae for such a length of time suggests that conditions in which his larvae were maintained were not conducive to settlement. The present study revealed that competent larvae can delay settlement for a considerable time in the absence of suitable stimuli and that if such stimuli are eventually not forthcoming the larvae die. No larvae survived longer than three weeks during the present study. As this aspect of their biology was not under investigation, no specific attempts were made to prolong larval life or to determine whether larvae which had survived for many weeks in the plankton were still capable of settlement. The fact that laboratory reared larvae will die rather than settle on unsuitable substrata does not lend support to any theory of survival strategy. It is to be expected that the chances of survival of a species are optimised by larvae which will eventually settle on an unsuitable

substratum rather than die. Crisp (1974) states: "Beyond a certain time the development process can no longer be dammed back and the larva proceeds to metamorphose under quite inappropriate conditions". This statement is almost certainly true of lecithotrophic barnacle cypris larvae which have limited energy reserves and thus will die when these energy reserves are exhausted. Such behaviour is consistent with good survival strategy and leads to the conclusion that the death of larvae in the laboratory situation is due to some other factor. However, the situation is totally different for planktotrophic larvae and it can be argued that, given a reasonably long larval existence, the point under discussion is, for such larvae, academic. If *Pomatoceros lamarckii* larvae can reach competence to settle at eight days and are able to survive for up to a further seven weeks the probability of failing to locate a suitable settlement site is very low. Failure to settle is more likely to be the result of other factors such as infection and in particular, predation. Pechenik (1990) makes the point that delay in metamorphosis can only be judged accurately when it can be shown with certainty that the larvae in a culture are ready to metamorphose and he suggests that larvae die in the laboratory because of unsuitable culture conditions or senescence.

The normal course of metamorphic events as observed in this study was found to be: metatrochophore ➡ settlement ➡ tube formation and loss of locomotory cilia ➡ formation of branchial crowns ➡ juvenile. The larval forms which Segrove (1941) described as stages of metamorphosis and which Føyn & Gjøen (1954) thought were larvae which had settled, secreted a tube and then left it, have frequently been observed during the course of the present study. Larvae which became tail-fixed while exploring substrata usually developed in this way. They failed to produce a

tube and there can be little doubt that these larvae were in some way abnormal. Eventually these abnormal larvae die and decompose without having attained the morphology of an adult worm. No evidence was found to support the view of Føyn & Gjøen (1954).

The settlement and metamorphosis of marine invertebrate larvae are complex processes, often involving the combination of many factors and responses to various stimuli. Crisp (1974) lists the factors important to the choice of settlement site as: texture, contour and permanence of surface, exposure to water flow, light, extremes of temperature and salinity, availability of food, danger from predators and competitors and chances of success in reproduction. It is questionable, however, whether factors such as danger from predators and chances of success in reproduction have any influence on the 'choice' of a settlement site by marine invertebrate larvae. As previously stated, such larvae are incapable of making choices, they respond to stimuli and do not possess the capability of considering whether a site is safe from predators or whether it has possibilities for reproduction. What can be said is that larvae respond to a stimulus or a combination of stimuli which cause them to settle. Because these stimuli are produced by or mediated by conspecific adults it is implicit that the settlement site is likely to provide the three basic requirements: food, minimal predation and reproductive success.

In many cases the most important stimulus to settle has been shown to be an adult-produced chemical cue adsorbed onto the substratum, but a combination of stimuli is often required (Jackson, 1986; Roberts *et al.*, 1991). Another factor in the settlement of the larvae of many, but not all, species of marine invertebrates is a biofilm. Because a biofilm is generally considered to be non-specific it is easy to overlook its importance in the

selection of a settlement site. Knight-Jones (1951) found that the majority of *Spirorbis borealis* larvae settled on surfaces which held conspecific adults, that settlement on glass or stone substrata only occurred on surfaces which had a biofilm and that settlement took place earlier on surfaces which held previously settled individuals than on bare, control surfaces. In addition to Knight-Jones (1951), many workers have noted the effect of biofilming on larval settlement (Zobell & Allen, 1935; Wilson, 1955, 1968; Meadows & Williams, 1963; Scheltema, 1974; Maki & Mitchell, 1985; Maki *et al.,* 1990). Wilson (1955), in his classical work on *Ophelia bicornis*, discovered that (unspecified) micro-organisms (presumably within the biofilm) were the most important stimulus to settle, saying: "The factor most active in inducing settlement of *Ophelia bicornis* larvae is the presence, on the sand grains, of living micro-organisms. The organisms must be neither too few nor too abundant".

Prior to the present study little was known about the way in which *Pomatoceros* species settled or what factors influenced the choice of settlement site. Moat (1985) noted that *P. triqueter* larvae settled preferentially on substrata having resident conspecifics, indicating a gregarious response, and his observations led him to believe that the contents of the anal vesicle played a major part in settlement.

In the present study *P. lamarckii* larvae preferentially settled on or close to adult tubes of the same species, supporting the findings of Moat (1985) and confirming that *Pomatoceros* species are gregarious as defined by Crisp (1990) when discussing barnacle larvae: "Gregariousness implies that the probability of cyprid settlement is increased by the presence of one of its own species, by part of one of its own species or by an extract therefrom". There was no clear evidence to suggest that larvae would

preferentially settle close to other, previously settled, larvae. There were occasions when larvae would settle alongside, or even on top of, previously settled larvae but also many occasions where the position of the initial settler(s) was avoided by later settlers in favour of other sites. This finding suggested that settlement was in response to a stimulus other than the presence of conspecifics. It was found that larvae did not settle on, or near locations on biofilmed slates from which newly settled wild juveniles had been removed. Mullineaux & Garland (1993), working on the responses of *Hydroides dianthus* larvae to boundary layer water-flow, found no gregarious response to newly settled conspecific juveniles whereas Scheltema *et al.*(1981), working on the same species, reported a gregarious response to adults.

If we consider the benefits of gregariousness to be: location of suitable safe habitats, maintenance of old breeding stocks and the formation of new ones (Knight-Jones, 1951), it can be argued that settlement on or close to an adult tube fulfils these requirements. The presence of healthy adults of the same species on a prospective site is a sure indication that the site is suitable for that particular species. It does not, however, follow that the presence of newly settled juveniles of the same species is an indication of a suitable settlement site. If the resident juveniles, for whatever reason, settled in an unsuitable situation such settlement could lead to a failure to reach a reproductive age or to a lack of opportunity to breed. Thus a gregarious response to other juveniles could be disadvantageous to later settlers. It would therefore appear advantageous that any gregarious response should, in the first instance, be to the presence of an adult. Such a response would imply that adults have a chemical stimulus which is not possessed by the juveniles, or that the composition of the surrounding

biofilm is made more 'attractive' by the presence of adults than by that of juveniles.

Working on *Phragmatopoma californica* , Jensen & Morse (1984) proposed that, by responding to cues on the anterior portions of the sand-tube, larvae possibly detect not only the presence of tube material but also the likelihood that the adult is alive and actively tube building. They were able to show that contact with the anterior ends of tubes resulted in a much greater incidence of metamorphosis than contact with the posterior ends of tubes or with sandstone. During the present study it was noted that exploring *Pomatoceros lamarckii* larvae would often investigate the anterior end of an adult tube before choosing a settlement site and it is quite possible that this exploration provided cues, as suggested by Jensen & Morse (1984) for *Phragmatopoma californica* , that the adult was alive and healthy. A settlement response to such a cue would ensure that larvae settled in a suitable habitat. The site preferred by early *P. lamarckii* settlers was often found to be the small hollows formed by the keel/sidewall junction and the substratum/sidewall junction close to the anterior end of the adult tube.

Settlement was preceded by distinctive settlement behaviour. Competent larvae often swam in an undulating path which took them alternately close to and away from the substratum. When a suitable substratum was encountered the typical larval search patterns of broad exploration, close exploration and inspection (Crisp, 1974) were followed. It was noted that most of the investigation of a substratum was carried out while gliding on the neurotroch cilia with the ventral surface of the well-defined 'head' region in contact with the substratum, rather than the apical cilia. It would appear, therefore, that the function of these apical sensory

cilia is largely restricted to the earlier pelagic phase and that they do not have a major role to play at the settlement stage.

If a satisfactory site was located following inspection, a settling larva would make a distinctive movement by sharply raising its body until the anterior half was at right-angles to the substratum. This sharp flexion of the body probably causes an explosive release of mucus from the mucous glands of the ventral shield epithelium. This degree of flexion can only be achieved if the posterior area of the body is attached to the substratum. Tail fixation alone would not suffice. The ventral surface of the posterior segments is well supplied with mucous glands and it has been noted that competent larvae are very sticky when handled. This stickiness suggests that the ventral, posterior surface of the larva is well coated with mucus at the time of inspection and that the adhesion provided by this mucus and a tail attachment, utilising mucus from the anal vesicle, is sufficient for the sharp flexion of the body to take place. Tail attachments and a tacky mucous coating should also prove to be advantageous in maintaining contact with the substratum while investigating prospective settlement sites in turbulent water conditions (see Nott, 1973).

Following the sharp flexion of the body the larva returned to the horizontal and was immediately firmly fixed. Fixation was achieved by a large pad of mucus in the region of the ventral shield epithelium. Rotational and horizontal movements designed to form the primary mucous tube were then commenced. The rotational movements followed a pattern of 360° in one direction followed by 360° in the other. The effect of these movements would be for mucus to be dragged, at each rotation, around the body from one side of the mucous pad to the other by the thoracic and abdominal chaetae. Here it would be re-integrated into the pad, thus

forming the primary tube. This method of tube formation bears many similarities to that described by Nott (1973) for *Spirorbis spirorbis*. The purpose of the longitudinal movements is probably to keep the tube open until the mucus becomes more viscous and also to spread the mucous pad over a greater longitudinal distance thus increasing the length of the primary tube.

As new tube is added and becomes semi-opaque with the advent of calcification, it is still possible to see the animal within. It was noted that, when the semi-opaque tube was *circa* 125 -175μm. in length the posterior end of the animal was still pointed, suggesting that the anal vesicle still contained mucus and that the anus remained displaced onto the dorsal surface. As the initial tube is often open at both ends, which can be demonstrated by pushing the animal out backwards, it is proposed that the remaining mucus contained in the anal vesicle is utilised to block the end of this tube to prevent the ingress of predators. It may also improve the effectiveness of the peristaltic movements of the animal in irrigating the tube to circulate water and remove waste products.

It has been shown that *Pomatoceros lamarckii* larvae will preferentially settle on or near conspecific adults and there is ample evidence that many species which adopt this gregarious response do so in response to a chemical stimulus emanating from the adult ((Knight-Jones, 1953; Crisp & Meadows, 1962, 1963; Wilson, 1970; Meadows & Campbell, 1972; Cameron & Hinegardner, 1974; Chia, 1978; Morse, 1990). There was, therefore, no reason to believe that *P. lamarckii* larvae did not respond to such a stimulus and exhaustive attempts were made to show the existence of a chemical stimulus but all attempts met with failure.

The majority of research workers must, when a promising hypothesis fails to be supported by experimental evidence, have cause to echo the sentiments of Charles Darwin which form the introductory quotation to this thesis: "All nature is perverse & will not do as I wish it" (Darwin, 1856, in Burkhardt & Smith, 1985). In these circumstances the first question is whether experimental technique was at fault, could it have been done any differently and have all avenues been explored? Secondly, in the case of larval settlement, the question; 'how many times should the experiment be repeated?' should also be asked. The density of larval settlement is extremely variable (Denny, 1988). There were numerous occasions when an apparently normal batch of larvae failed to settle in what appeared to be ideal circumstances and therefore the possibility can exist that an experiment failed simply because the larvae were not competent to settle. In some circumstances the competence of the larvae which were used in a failed experiment could be judged by introducing them to a substratum which is known to be highly attractive, e.g. a conspecific adult tube. If a reasonable proportion of the larvae settled on this substratum non-competence could be ruled out as a reason for failure of the experiment. This check was used where possible in the present study and the conclusion reached was that *Pomatoceros lamarckii* larvae did not respond to any form of adult extract. This finding implies that either a chemical stimulus does not exist, or that it was not extracted in an active form by the methods used.

Meadows and Campbell (1972) suggest that settling larvae may simply be responding adaptively to metabolic by-products of excretory products that are leaked into the environment. The possibility that competent larvae might be responding to a stimulus carried in the exhalent irrigation flow,

emitted from the anterior end of the adult tube, was investigated without success during the present study. Similarly, swabs taken from various parts of the tube and animal and then smeared onto suitable substrata also failed to elicit settlement.

Although a remote possibility remained that it might, in time, be possible to extract a chemical cue the available evidence suggested that this line of research was no longer worth pursuing. The fact remained, that *Pomatoceros lamarckii* larvae settled readily on adult tubes and on the surrounding substratum. It was also observed that, during the course of the attempts to discover a chemical cue, larvae often settled on biofilmed surfaces in preference to surfaces which had merely been treated with *P. lamarckii* extracts. Could it be that the larvae were responding to the biofilm? There would appear to be no logical reason why this response should be so. A non-specific stimulus should not, in theory, lead to gregarious settlement, but was the biofilm necessarily non-specific? Did the presence of adult *P. lamarckii* modify the biofilm to make it more attractive to settling larvae? The possibility that biofilming could play a major role in settlement was strengthened by the discovery of those pebbles on the beach at Dinas Dinlle, Lleyn Peninsula, which showed no visible traces of adult *Pomatoceros* species, but which held large aggregations of live, recently settled, juveniles. There is also supportive evidence in the literature that biofilming plays an important role in the settlement of some invertebrate species; (Zobell & Allen, 1935; Knight-Jones, 1951; Wilson, 1955; Meadows & Williams, 1963; Cameron & Hinegardner, 1974; Scheltema, 1974; Kirchman *et al.*, 1982; Kirchman & Graham, 1982; Bonar *et al.*, 1986; Maki *et al.*, 1990; Roberts *et al.*, 1991).

Settlement experiments carried out on slate substrata which had been biofilmed in a variety of ways provided conclusive evidence that biofilming was important in the settlement process of *Pomatoceros lamarckii* larvae. These experiments also showed that substrata which had been biofilmed in the presence of adult *P. lamarckii* were more attractive to settlers than those which had not. The results of the substratum acceptability experiments, where the larvae were given no choice between substrata, showed an almost equal preference for adult mediated biofilms which had been formed in static, fine filtered and ultra-violet irradiated seawater (FFSW) and those which had been formed in adult mediated running seawater (RSW). When exposed to the two types of treated substrata, those substrata which had been adult/FFSW biofilmed were significantly more attractive than adult/RSW biofilmed substrata. These findings strongly supported the hypothesis that the biofilm was, in some way, modified by the presence of conspecific adults.

It remained to discover whether any difference, which might account for the distribution of the settled larvae, could be detected in experimental biofilms. Microscope slides which had been biofilmed in the same way as the experimental slate substrata, when stained for the presence of bacteria, showed that the bacterial film on the adult/FFSW biofilmed slides was considerably more densely stained than that on any of the others. This difference in bacterial filming was evident to the naked eye. Examination under a microscope revealed that the adult/FFSW biofilm had a great number of bacteria of a type which was absent, or present only in low numbers, on the other slides. Further work needs to be done before it can be shown that these bacteria are responsible for the enhanced settlement on substrata which have been biofilmed in the presence of adults, but this

finding is not without precedent. Wilson (1955) found that the most important factor in the settlement of *Ophelia bicornis* larvae was the presence of living micro-organisms on the sand grains and Hadfield (pers. comm.) working on the settlement of *Hydroides elegans*, found that the larvae settled highly preferentially on biofilmed surfaces. This preferential settlement appeared to be closely related to the presence of a small, rod-shaped bacterium. Christensen *et al.* (1985) showed that *Pseudomonas* species elaborates two different extracellular polysaccharides in batch culture. One polysaccharide was produced only during exponential growth, the other was released at the end of the exponential phase and in the stationary phase. This finding opens up the possibility that settlement of invertebrate larvae could be influenced by bacteria or their exopolymers at specific stages of the bacterial growth cycle.

It is not immediately obvious why larvae should settle in greater numbers on adult/FFSW biofilmed substrata than on adult/RSW biofilmed substrata when given a choice between the two, as they settled in approximately even numbers on each type of biofilmed surface if presented with no choice. If it is accepted that bacteria or their exopolymers are the causative factor in the stimulus to settle then the answer may lie in the conditions in which the biofilming took place. The adult/FFSW biofilming took place in a small volume of, initially, bacteria-free static water. The build up of faeces and other waste products would quickly lead to high concentrations of bacteria on the surfaces in this water. Although the RSW initially contained many more bacteria than the FFSW, the adult/RSW biofilming took place in a much larger volume of water which was constantly being replenished. This situation would lead to a much slower build up of bacteria on the surfaces being biofilmed.

The low settlement on the other biofilmed substrata which were tested (slates biofilmed in FFSW + food algae and slates biofilmed in RSW only) was again consistent with the small number of bacteria found on each surface. It was to be expected that these surfaces would have low numbers of bacteria. The build-up of any bacteria in the container holding FFSW + food algae would be slow in the absence of quantities of faecal material and other contaminants. The RSW only container had a continuous flow of RSW without the presence of *Pomatoceros lamarckii* adults to provide a source of the bacteria which are postulated to be the major factor in settlement.

Interestingly, the substrata which were biofilmed in RSW only were, from time to time, found to have wild settlers when examined prior to use in settlement experiments. These larvae (which were removed prior to experimental use) obviously found the substrata sufficiently attractive to settle. At the termination of experiments using these substrata it was found, almost without exception, that settled larvae had ignored the locations of the wild settlers and that very few larvae had settled on these substrata at all. There are two points to be made here; the first is that the cultured larvae showed no response to the traces of other juveniles, thus supporting the argument that it is beneficial, in survival terms, for larvae to settle in the presence of adults, but not for them to settle in the presence of other juveniles. Secondly, the substrata biofilmed only in RSW were, in this instance, attractive to larvae which were offered no other alternatives but when exposed to a variety of biofilmed surfaces the larvae responded to what would appear to be the most attractive option. This finding suggests that, having encountered a weakly or marginally attractive surface,

competent larvae will be stimulated to settle if no more potent stimulus is immediately available.

The discovery at Dinas Dinlle of large numbers of juvenile *Pomatoceros lamarckii* which had settled on pebbles holding no adults therefore appears to be an anomaly. If the stimulus to settle was produced by the presence of bacteria why was settlement confined only to certain pebbles? This situation, of the colonisation of some pebbles in preference to other, apparently similar ones, is to be seen at any site where *Pomatoceros* species are abundant. Pebbles bearing large numbers of juveniles alone have, during the present study, only been found at Dinas Dinlle. The answer to this question is not clear at present and research should be carried out to attempt to reveal any differences in the structure, micro-topography and biofilm of adjacent settled and unsettled pebbles. A further possibility worthy of investigation is that rock type could influence the types of micro-organism which comprise a biofilm. A pebble beach is normally composed of a variety of rock types and a comparatively soft, calcareous pebble may support a very different biofilm to that of a smooth, hard granitic pebble. Another interesting possibility is that colonies of bacteria on adjacent stones may be in different phases of growth, resulting in the production of different exopolymers (Christensen *et al.*, 1985) only one of which might be capable of inducing a settlement response.

If specific bacteria provide the major stimulus for the inducement of settlement of *P. lamarckii* larvae (and the available evidence suggests that this premise is correct), then these bacteria must exist, perhaps in small numbers, throughout the same part of the marine environment as *P. lamarckii*. As suggested above, they may also be limited to particular rock types by a variety of factors. We have seen that substrata biofilmed only in

RSW will attract settlers when small numbers of bacteria are present. It is, therefore, highly likely that competent larvae will settle on a marginally attractive surface in the absence of a more positive cue and, given two marginally attractive surfaces, the surface with the stronger stimulus will attract more settlers.

Although the case has been made for larvae not being attracted to settle by the presence of newly settled juveniles, we must here re-consider the scenario proposed by Crisp (1974) who suggested that larva will metamorphose under quite inappropriate conditions when the development process can no longer be repressed. Although larvae which fail to find an acceptable substratum under laboratory conditions die rather than settle on an unsuitable substratum, there is no evidence to suggest that this situation occurs in nature and the basic tenets of the survival of the species mitigate against it. Therefore it is more than likely that a larva which has reached the development stage described by Crisp (1974) will respond to the least unfavourable option/stimulus available. This option/stimulus could well be the presence of isolated juveniles on a substratum. These isolated juveniles, by the same precept, could have settled in response to the weak stimulus provided by a small number of suitable bacteria. A further point in support of the argument for settlement without the presence of conspecific adults is that the ability of a species to colonise new territory, or extend to the environmental limits of its range, would be severely impaired if larvae were only to settle in the presence of adults.

As yet few researchers have investigated the effects of biofilms on the settlement of marine invertebrate larvae but there is growing evidence that this effect may be a productive area of research (Kirchman & Graham, 1982; Bonar *et al.*, 1986; Maki *et al.*, 1990). In the case of *Pomatoceros*

lamarckii much work remains to be done in determining the strain(s) of bacteria responsible for providing the stimulus to settle and the relationship of these bacteria to adult *Pomatoceros lamarckii.* Blenkinsopp & Costerton (1991) point out that, in the complex glycocalyx (matrix) of extracellular polymeric substances produced by micro-organisms, the metabolite of one organism may be the nutrient of another. In this case one species may only flourish in the presence of another. The situation is further complicated by the fact that, following initial colonisation of a substratum by bacteria, complex biofilm communities develop with the advent of 'various planktonic cell types'. Blenkinsopp & Costerton (1991) refer to such a community as a "consortium". Meadows & Campbell (1972) state that the abundance of diatoms, blue-green and green algae may be equal to that of bacteria on the surface of sand grains. It may well prove to be that *P. lamarckii* settlement is in response to chemical cues produced by and within such a consortium.

Surface contour was found to have a secondary effect on *P. lamarckii* settlement. During the course of the present study it was found that, when using slates with shallow pits, larvae consistently found the angle between the base and side of the pits to be a particularly attractive settlement site. In the definitive biofilming experiments the pits again proved attractive, but only if the biofilm was attractive. Similarly, in other experiments using slates without pits, larvae would often settle preferentially in the shallow grooves forming the grid which was engraved on the slates to assist in precise location of settled larvae. Again, this preferential settlement would only occur if the biofilm was attractive.

Both the tubeworm, *Pomatoceros lamarckii,* and the macroalga, *Laminaria digitata,* have, in the present study, been shown to employ mechanical adhesion to attach themselves to substrata. In the case of *P. lamarckii* the mechanical adhesion was attained by the very fluid nature of the newly formed mucus/calcium carbonate tube material which is able to conform precisely to every small variation of surface topography. *L. digitata* adheres by the ability of the terminal, unicellular rhizoids, which form the contact area between the haptera and a substratum, to penetrate and conform exactly to every irregularity of the surface to which it is attached. Attachment is enhanced and strengthened by a mucous coating of the rhizoids, which appears to be a common feature of many of the macroalgae (Moss, 1973; Tovey & Moss, 1978; Hardy & Moss, 1979; Fletcher, 1980).

In the present study the tenacity (force per unit area) of *L. digitata* has been shown to be appreciably less than that of *P. lamarckii* . There are three reasons why a lower tenacity might be expected. The first of these reasons is that the test substrata were all relatively smooth, offering little in terms of surface contour which could be fully exploited by the ability of the rhizoids to conform to irregularities in the substratum. Secondly, owing to the way in which the individual haptera grow in order to resist a strongly uni-directional force, it was found to be difficult to arrange a direction of pull which did not induce a certain amount of peeling of the hapteron prior to detachment. Peeling markedly reduces the forces of detachment. Grenon & Walker (1981), working with the limpet, *Patella vulgata,* calculated that peeling resulted in a tenacity ≈75% of that of a perpendicular pull. Consequently, in those cases during the present study where peeling of *L. digitata* haptera was seen to occur, only minimal

tenacity figures could be calculated. Finally, even when a pull was set up to give an optimum result, the hapteron itself always failed below 6.5 x 10^5 N m^{-2} leaving the tissues of its base firmly attached to the substratum. This cohesive failure in the plant tissues probably provides the best explanation for the difference in tenacities between *Pomatoceros lamarckii* and *Laminaria digitata* , as it is unreasonable to assume that soft, cellular plant tissue should possess a similar tensile strength to that of a hard, mainly inorganic, tube.

Comparison with other sessile organisms (Table 1) shows *P. lamarckii* to have a considerably higher tenacity than the semi-mobile barnacle, *Balanus balanoides*. *L. digitata* is comparable in tenacity to mobile organisms which employ a 'temporary' adhesive (Crisp, 1972).

L. digitata would appear to have adequate tenacity to meet the stresses of its environment (it would be surprising if it did not do so!). As stipe and blade grow, offering more resistance to water flow and thus increasing chances of detachment, so more and larger haptera are produced to anchor the plant firmly to the substratum. Failure normally occurs at the stipe, a fact which can be confirmed by an examination of any kelp bed where holdfasts bearing the short stubs of broken stipes are not uncommon. Such breakages may be attributed to senescence as they normally occur among older plants. In the normal course of events the flexibility of the *L. digitata* stipe and blade enables the plant to minimise the forces acting upon the holdfast and the plant as a whole. Occasionally, whole *L. digitata* sporophytes, including the holdfast, are to be found on the shore after a severe storm. Examination of such holdfasts reveals that the haptera attached to encrusting organisms, e.g. barnacles, which have subsequently died and decomposed, forming a weak interface between the substratum

and the holdfast. It is the failure of this interface and not lack of strength of the holdfast which has resulted in the plant becoming detached.

A *Pomatoceros lamarckii* tube appears to have a tenacity well in excess of its requirements (Table 1). Its small size, low profile and relatively streamlined shape should ensure that quite a low tenacity would enable it to maintain contact with its chosen substratum. If it can be assumed that the calcareous tube evolved for other reasons, then high tenacity can be seen as a by-product of tube formation. Two obvious purposes for the tube immediately spring to mind, the first of which is protection from predators, the second being the conferred advantage of being able to retain a self-contained environment for short periods of time when out of water. The latter facilitates the occupation of a niche in the littoral zone and has probably allowed *Pomatoceros* species to extend their range from the sub-littoral zone to the mid-littoral.

Table 1: A comparison of the tenacities of a variety of marine organisms (N m^{-2} x 10^5 ± SD).
(m) = mobile. (s) = semi-mobile. (i) = immobile.

Species	Tenacity	Reference
Tubeworms (i):		
Pomatoceros lamarckii	24.75 ± 6.63	Present study
Barnacles (s):		
Balanus balanoides	9.3 ± 4.9	Yule & Walker, (1984)
Balanus glandula [1]	4.17 ± 2.31	Denny *et al.*, (1985)
Mussels (s):		
Mytilus edulis	5.62 ± 0.77	Young & Crisp, (1981)
Limpets (m):		
Patella cochlear [2]	5.2 ± 0.9	Branch & Marsh, (1978)
Patella vulgata	2.3 ± 0.2	Grenon & Walker, (1981)
Snails (m):		
Littorina scutulata	1.05 ± 0.85	Millar, (1974)
Thais emarginata	0.72 ± 0.37	Millar, (1974)
Anemones (s):		
Actinia equina	4.6 ± 1.26	Young *et al.*, (1988)
Macroalgae (i):		
Laminaria digitata	4.22 ± 2.7	Present study

[1] The figure quoted is that for shear strength.
[2] A tropical species from a high energy, wave-swept environment.

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Appendix 1

Rearing *Pomatoceros lamarckii* Adults in Glass Tubes

Pomatoceros species can be induced to adopt glass tubes of suitable bore (Dales, 1981) thus facilitating observations which would not normally be possible. The bore of the glass tube is quite critical; if it is too large the animal works its way out and if too small it is difficult to insert the animal without injury and/or the animal becoming jammed within the tube.

It is not difficult to keep animals in glass tubes for short periods of time but unless an animal has truly adopted the tube any observations made may be of aberrant behaviour. During the present study *Pomatoceros lamarckii* adults were introduced to glass tubes in order to be able to study tube formation and movement of the animal within the tube. Of the animals introduced to glass tubes only two lived for appreciable lengths of time and could be said to be well established. The first successful animal lived for 85 days in a glass tube during which time it produced a short, calcareous extension and a calcareous end-plate. The second successful animal lived for over thirteen months during which time it produced a sufficiently long calcareous tube to enclose the worm completely.

The best shape for a glass tube was found, by trial and error, for it to be slightly tapered along its length and very slightly belled at one end (the anterior end). Such tubes may be made from the thin, tip section of a

Pasteur pipette using normal glass-blowing techniques. Sharp ends should be smoothed by briefly melting in a Bunsen flame but the tube should remain open at both ends to facilitate insertion of the animal. Several tubes of slightly different internal diameters should be made to allow a choice of sizes for any particular animal. Tubes should be sufficiently long to accommodate the whole animal.

Animals were removed from their natural tubes by gradually breaking off pieces of the posterior end of the tube until it was sufficiently short and straight to be able to push the animal out backwards by applying gentle pressure on the operculum with a wire of suitable diameter. Using a pair of fine forceps, the animal was then inserted into a glass tube of suitable bore by introducing the posterior segments into the bell-end of the tube and gradually easing the animal into the tube until only the branchial crowns protruded. Care should be taken not to damage the animal during the process of insertion as such damage usually results in death.

If the tube size had been correctly judged the animal would remain in the tube but if the bore was too large the regular contractions of the body segments would soon drive the animal out of the tube. In such cases the animals were re-inserted into tubes of smaller bore. Having inserted an animal into a tube of suitable proportions, it was occasionally found helpful to insert a small plug of Plasticine modelling clay into the posterior end of the tube. The plug was pushed up the tube until it came into contact with the posterior segments of the animal when it was in the feeding position (with branchial crowns extended). As the smooth surface of a glass tube provides little purchase for the thoracic chaetae the animal can have difficulty in moving forward into the feeding position if it moves too far down the tube. The plug prevented excessive backward movement from

occurring and also provided a stable structure against which the animal could push in order to assist its forward movement. Animals which had been successfully inserted into glass tubes were maintained in correct orientation (ventral surface uppermost) by securing the tube to 20 x 50 x 3.5mm. slate substrata with a small piece of Plasticine. Finally these slates were placed in containers of fine filtered and ultra-violet irradiated seawater containing a 1:1 mixture of unicellular food algae (*Tetraselmis chui and Rhinomonas reticulata*) at 200 cells μl^{-1}. The containers were maintained in a controlled environment cabinet at 17°C with a 12:12 h. light:dark regime.

Animals which successfully adopted glass tubes first began to lay down new calcareous tube around the rim of the glass tube *circa* four days after introduction. The first indications of this calcareous tube appeared in the area of the keel-forming fold of the ventral part of the collar (Fig. 1). At the same time an end-plate also began to form towards the posterior end of the glass tube. Initially this end-plate consisted of a semi-opaque sheet of mucus which was moulded to the shape of the posterior abdominal segments. By day 25 the end-plate was well calcified and the beginnings of a keel could be seen on the new tube which was also starting to attach to the substratum (Fig. 2). Even when well calcified the end-plate appeared to consist of a calcified grid pierced by small holes (Fig. 3, chapter 3). Gentle probing with a stiff bristle showed that the end plate was, in fact, a complete sheet of mucus which was only partly calcified.

During tube formation the animal must be well forward, in the feeding position, with the collar over the end of the tube. The end-plate was always formed in such a position that the animal was constricted when it withdrew into the tube which suggests that it was formed at the same

time as new tube was being formed, while the animal was in the forward position. At 47 days the animal had a well established calcareous tube which was firmly attached to the substratum and had also produced a substantial calcareous lining to the anterior part of the glass tube (Fig. 3). This finding suggests that either tube formation is not restricted to the immediate area of the collar where it folds over the end of the tube, or that some of the very fluid tube material is squeezed backwards down the tube wall by the movements of the animal.

At 82 days the longest lived animal produced a second end plate immediately anterior to the first (Fig. 4). Animals which have been grown on glass during the present study (in order to be able to view the animal from the underside) have been seen to produce calcareous end-plates at irregular intervals within their tubes. These end-plates undoubtedly serve the purpose of preventing the ingress of predators as the weaker, older tube begins to disintegrate. They may also be necessary to enable effective irrigation of the tube to take place as without such end-plates the peristaltic movements of the animal may be less effective in removing waste products and circulating water around the animal. Animals in natural tubes have not been observed to produce end-plates in close proximity to each other, but if the tube is broken at the posterior end the animal will effect a repair by building an end-plate within a few days.

Finally, it is worth noting that the technique for growing *Pomatoceros* species in glass tubes can be equally effective in transferring animals to other natural tubes, to which they adapt quite easily if tubes of suitable internal dimensions are used.

Figure 1.

Light micrograph of a *Pomatoceros lamarckii* adult 4 days after insertion into a glass tube. Calcareous tube material (j) has just started to be secreted onto the anterior edge of the glass tube. Scale bar...1 mm.

Figure 2.

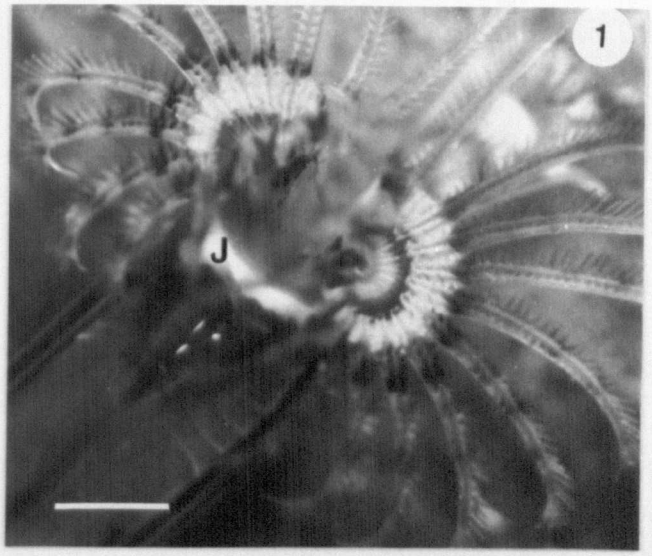
Light micrograph of a *Pomatoceros lamarckii* adult 25 days after insertion into a glass tube. The tube (j) is extending and the keel is just starting to form. An end-plate (v) has been built into the posterior part of the tube. The white band which can be seen on the animal is a band of white pigmentation on the branchial crowns. Scale bar...2 mm.

Figure 3.

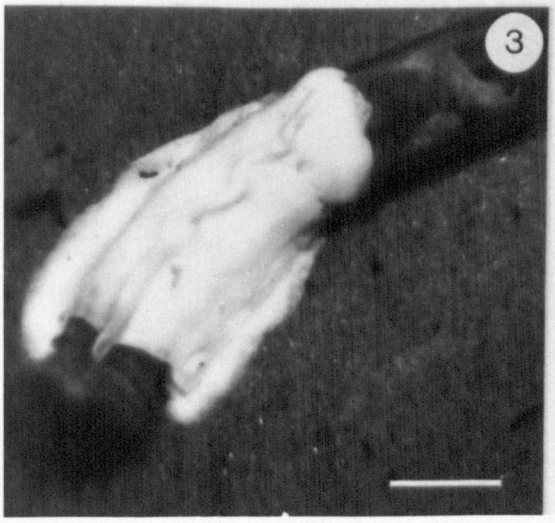
Light micrograph of a *Pomatoceros lamarckii* adult 47 days after insertion into a glass tube. The tube is now quite long and firmly attached to the substratum. Scale bar...2 μm.

Figure 4.

Light micrograph of a *Pomatoceros lamarckii* end-plate 82 days after insertion of the adult into a glass tube. A second end-plate has been formed immediately anterior to the first. The darker, right-hand vee of calcareous material is the upper part of the original end-plate. Scale bar...600μm.


1
J


2
V
J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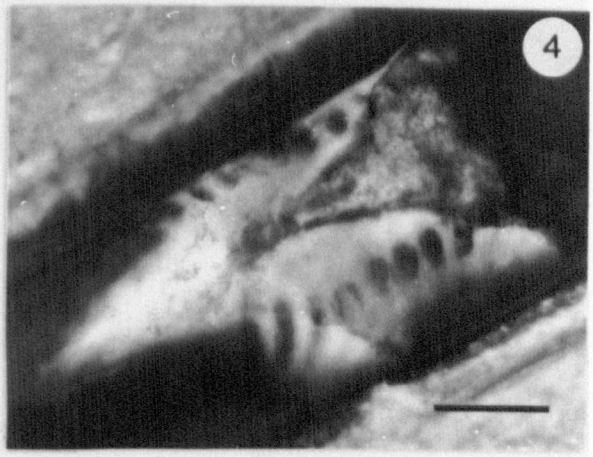
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Appendix 2

Data Relating to Tables 1 - 4, Chapter 7

Table 1: Data for *Pomatoceros lamarckii*, Tables 1 & 2. **Sub.** = substratum. **SS.** = smooth slate. **RS.** = rough slate. * = clean pull

Substratum	Pulling force (N)	Area of tube removed by pull (sq.mm.)	Area of sub. removed by pull (sq.mm)	Tenacity ($N\ m^{-2} \times 10^5$)
SS.	7.12	18.35	1.33	3.9
SS.	4.66	2.79	-	16.7
SS.	5.1	10.39	2.31	4.9
SS.	16.67	19.52	0.78	8.5
SS.	19.6	6.49	-	30.2 *
SS.	9.56	8.99	2.36	10.6
SS.	14.22	10.04	1.32	14.2
SS.	15.69	8.38	2.88	18.7
SS.	4.66	2.44	-	19.0*
SS.	9.22	12.03	0.5	7.7
SS.	6.67	1.99	-	33.5*

Table 1. continued

Substratum	Pulling force (N)	Area of tube removed by pull (sq.mm.)	Area of sub. removed by pull (sq.mm)	Tenacity (N m^{-2} x 10^5)
SS.	5.98	14.03	2.22	4.3
SS.	13.34	4.78	-	27.9*
SS.	3.92	6.8	1.29	5.8
SS.	11.5	5.43	-	21.2*
RS.	14.32	7.03	-	20.4*
RS.	10.68	8.79	1.41	12.2
RS.	14.71	11.49	0.33	12.8
RS.	11.03	4.72	-	23.4*
Stone	8.09	6.09	3.49	13.3
Stone	5.59	1.99	-	28.1*
Stone	6.7	4.12	-	16.3*
Stone	10.3	13.69	3.89	7.52
wood	0.98	20.61	9.54	0.48

Table 2: Data for *Laminaria digitata*, Tables 3 & 4.
Sub. = substratum. **SS.** = smooth slate. **RS.** = rough slate.
* = clean pull

Substratum	Pulling force (N)	Area of tube removed by pull (sq.mm.)	Area of sub. removed by pull (sq.mm)	Tenacity ($N\ m^{-2} \times 10^5$)
SS.	0.24	0.38	-	6.3*
SS.	3.12	11.42	-	2.7*
SS.	0.79	8.84	-	0.9*
SS.	0.96	4.53	-	2.1*
SS.	7.16	15.45	-	4.6*
SS.	4.71	13.93	-	3.4*
SS.	2.69	16.72	2.38	1.6
SS.	5.71	56.11	33.86	1.0
SS.	2.5	16.01	2.2	1.6
RS.	6.52	10.63	-	6.13
RS.	14.46	35.77	1.1	4.0
RS.	3.87	17.02	-	2.3