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Genetic and phenotypic variation in two marine bryozoans.

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GENETIC AND PHENOTYPIC VARIATION IN TWO MARINE BRYOZOANS

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by
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This thesis is dedicated to the memory of

Martin (Mart) Lees

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SUMMARY

Chronic copper toxicity was found to result in growth hormesis in both *C. hyalina* and *E. pilosa*. Tolerance levels of the bryozoans to copper contamination were found to be high in comparison with those of other marine organisms. Generally, both genotype and copper dosage affected growth and sexual functions, but genotype had no affect on organism response to copper. Nonetheless, large differences in responses were detected between the two *E. pilosa* populations examined.

Variation in tentacle number of *C. hyalina* colonies was found to be very limited and dietary regime was not observed to have a marked influence upon this trait. In contrast, colonies of *E. pilosa* were found to produce more tentacles per lophophore in optimal dietary conditions. Significant differences were detected between two *E. pilosa* populations in astogeny, sexual maturity and tentacle number.

Laboratory experiments designed to identify the cue for induction of extended (long) medium proximal spines in *E. pilosa* colonies were unsuccessful. Differences in spine growth were again identified between the two *E. pilosa* populations from contrasting sites. Reciprocal transplantation demonstrated that 'long' spine formation was triggered in colonies previously possessing only 'short' spines and vice versa. Flume observations of particle path trajectories imply that spine formation may result in near-colony flow conditions which are more favourable to feeding in high flow velocities.

Demographic analysis using the RAPD technique for both *C. hyalina* and *E. pilosa* indicate population structuring corresponding to their contrasting modes of larval dispersal. Populations of *C. hyalina* appear to exhibit considerable genetic differentiation over distances as small as 100 m, whereas *E. pilosa* is characterised by high levels of genetic heterogeneity over much larger spatial scales. *E. pilosa* population differentiation is observed at a site some 80 Km distance, which may be a consequence of hydrographic features.

Evidence from analysis of mtDNA (COI) and observations on reproductive isolation and morphological differentiation indicate high levels of cryptic speciation amongst globally distributed populations of *C. hyalina*. It is suggested that the Chilean *C. hyalina* population is sufficiently different from all other populations examined to be considered a separate species.

CONTENTS

CHAPTER 1.

GENERAL INTRODUCTION

Bryozoans are filter-feeding, aquatic, colony-forming coelomates, of which there are over 5000 extant species that are nearly exclusively sedentary (Ryland, 1970; Horowitz & Pachut, 1994; for exceptions see, Cook & Chimonides, 1986; Peck *et al.*, 1995). Colonies are formed from the asexual budding of a primary zooid, ancestrula or statoblast. Ancestrulae are the product of metamorphosis of sexually produced larvae, statoblasts are the asexually produced resting phase of the freshwater class the Phylactolaemata. The phylum Bryozoa is comprised of three main classes, the Phylactolaemata which are exclusively freshwater, the Stenolaemata which is comprised of only one extant order, the Cyclostomata, and finally the Gymnolaemata. The Gymnolaemata are predominantly marine and form two orders, the Ctenostomata and the more recent Cheilostomata. Two taxonomic sub-divisions or 'sub-orders' are made within the Cheilostomata, the Anasca and Ascophora. The Ascophora possess highly calcified frontal walls and rely on an ascus (underlying sac) to regulate internal hydrostatic pressure. Anasca, on the other hand, have calcified side walls and use a frontal membrane for pressure regulation.

The bryozoan species, *Celleporella hyalina* (L.) and *Electra pilosa* (L.), used in this study, belong to Ascophora and Anasca, respectively. Both species have cosmopolitan distributions in temperate waters, often occurring as epiphytes of marine macroalgae, although frequently are found to occur on other substrata (Marcus, 1938; Ryland & Hayward, 1977; Hayward & Ryland, 1979; Morris, 1980). Around the coast of Britain *C. hyalina* is most commonly found associated with the kelp *Laminaria saccharina*, and to a lesser extent with the fucoid *Fucus serratus*. *E. pilosa* is extremely catholic in its substrata choice, but is also commonly associated with *F. serratus* (Ryland & Hayward, 1977; Cancino, 1986). Unusually for the cheilostomes, *E. pilosa* possesses a pelagic, cyphonautes larvae, which is thought to be present in the plankton for a number of weeks (Atkins,1955; Schopf, 1977). In contrast, *C. hyalina* is a more typical larval brooder, releasing short-lived, non-feeding, lecithotrophic larvae (Zimmer & Woollacott, 1977). The variation in

larval mode between these two cheilostome species raises questions regarding the degree of dispersal achieved and its consequent effects on population size and structure (Jackson & Coates, 1986). Unlike some other modular organisms, colony fragmentation is thought to be rare and not a significant means of dispersal for bryozoans (Hughes, 1983; Seed & Hughes, 1993; but see Winston, 1983).

The modular construction of colonial organisms such as bryozoans (Hughes, 1989), can present an array of characters suitable for biometric study. The formation of morphologically distinct sexual and feeding zooids in *C. hyalina* makes it particularly well suited to studies of relative sexual allocation (Hughes & Hunter, 1986b; Hunter & Hughes, 1993b, 1995; Yund & McCartney 1994; Hunter *et al.*, 1996; McCartney, 1997). Male and female zooids are generally budded frontally, overlying the feeding autozooids. However, male zooids can also occur amongst the autozooids (basal males) and autozooids may be frontally budded as well (Cancino, 1983). Colonies of *E. pilosa,* in contrast, do not possess separate sexual zooids and are generally comprised of a unilaminar sheet of feeding autozooids, which are protandrously hermaphrodite (Hughes,1986). The autozooids of all bryozoans possess a crown of ciliated tentacles which can be withdrawn inside the zooid module. Feeding currents are generated by the action of lateral cilia along the length of the tentacles (Ryland, 1976). Particles are drawn down into the lophophore and between the tentacles where they can be intercepted by the lateral cilia and subsequently transported towards the mouth (Best & Thorpe, 1986a). In *E. pilosa,* the lophophore of the autozooids performs a second function, that of sperm release into the water column. Sperm are liberated via a pore in either of the two dorsomedial tentacles (Silén, 1966). Sperm may then be captured by the tentacles of a conspecific which liberates mature ova from the inter-tentacular organ situated just beneath the lophophore (Silén, 1966). Fertilisation is thought to occur immediately outside the inter-tentacular organ (Silén, 1966). Male zooids of *C. hyalina* are non-feeding but possess a lophophore for the purpose of sperm release (Cancino, 1983; P. Manríquez pers. comm.). In this species, fertilisation is thought to be internal and the resulting embryo is brooded for 2 to 3 weeks before release (Hughes, 1987; P. Manríquez pers. comm.). Laboratory based culture has been established for both species, enabling the implementation of strict experimental regimes (Hunter & Hughes, 1991; Bayer *et al.*, 1994).

The modularity of bryozoans presents the opportunity to produce clonal replicates (ramets) of a single genotype (genet) (Harper, 1977). Subjecting ramets of several genotypes to a range of environmental conditions is an effective manner in which to investigate the interaction between genotype and environment (Stearns, 1992). Evidence of such interaction is crucial to understanding the persistence of sexual reproduction in the majority of multi-cellular organisms in spite of its inherent costs (Williams, 1975; Lewis, 1987). Identification of genotype-environment interaction provides support for the 'Tangled Bank' hypothesis for the evolutionary significance of sexual reproduction (Bell, 1982). Bell (1982) theorised that the release of sexually produced offspring with diverse genotypes, into a heterogeneous environment, should reduce competition between siblings that would normally occur between asexually produced progeny.

The virtues of clonal organisms as biological monitors have also been extolled (Calow, 1992; Forbes & Depledge, 1992; Lovett Doust *et al.*, 1993). This is again largely due to the fact that genotype (withholding somatic mutations) can be factored out of phenotypic response providing less inherent variation. An understanding of how pollutants affect a wide range of taxa is an essential criterion towards enabling accurate predictions to be made about how specific pollution incidents will affect local ecosystems (Forbes & Forbes, 1994).

From an anthropogenic perspective bryozoans might appear to be inconsequential. However, members of this phylum are worthy of note for a number of reasons. Bryozoans form an important component of the bio-fouling community throughout the world's oceans (Gordon & Mawatari, 1992). Bio-fouling is thought to cost the shipping industry alone, $1000 (US) billion a year (Clark *et al.*, 1988). Paradoxically, the search for novel compounds amongst the Bryozoa have yielded potential anti-fouling compounds, as well as, pharmacologically active substances (Clare, 1995; Prinsep & Morris 1996). For example, bryostatin 1, a protein kinase C modulator, has been isolated and tested as an anti-cancer agent (Newman, 1996). The phylum also represents one of the three dominant groups of Paleozoic fossils, and are abundant from Ordovician to Recent (McKinney & Jackson, 1989). This makes them excellent subjects for the study of evolution using the fossil record.

In addition, bryozoans form part of a suite of marine suspension feeding organisms that play a paramount role in littoral marine food webs (Gili & Coma,

1998), the littoral zone being one of the oceans most productive areas (Barnes & Hughes, 1988). In colder marine environments bryozoans can be very abundant, for example in Antarctic waters benthic accumulations of dead bryozoans and sponges can form a layer 5 cm deep (Androsova, 1973; cited in Winston, 1983), the former making up the largest portion of the living biomass in these waters (Moyano, 1979; Winston & Heimberg, 1988). The relatively high incidence of both intra- and inter-specific competition amongst bryozoans on some substrata have led to their use in a considerable number of studies investigating competition amongst sessile fauna for available resources. (Stebbing,1973a, 1973b; Buss, 1979, 1981; Wood & Seed, 1980; Seed & O'Connor, 1981; Best & Thorpe, 1986a, 1986b; Okamura, 1988; Turner & Todd, 1994). The formation of competitive networks is thought to strongly influence the structure and diversity of sessile marine species assemblages (Jackson, 1979).

Bryozoans offer a great deal of potential as experimental animals, through modularity, small size and sedentary habit. This study aims to investigate a range of ecological and life history questions using these attributes to best advantage.

The following research examines the genetic structure of populations over distance and establishes the extent to which genotype affects phenotypic variation under varying experimental regimes. Evidence of genotype-environment interactions may help in the understanding of local adaption and the maintenance of sexual reproduction (Stearns, 1989,1992). Only a very limited body of evidence exists of such interactions in the animal kingdom (Hughes, 1992). The importance of a variety of stress inducing factors is examined in the production of different morphological characters. Copper toxicity (Chapter 2), dietary variation (Chapter 3) and wave related stress (Chapter 4) are used as environmental variables. An assessment of the implications of larval mode in relation to population genetic structure is undertaken for both *E. pilosa* and *C. hyalina* (Chapter 5). Finally, the likelihood of speciation in *C. hyalina* over its extensive geographical range is examined (Chapter 6).

CHAPTER 2.

THE EFFECT OF CHRONIC COPPER EXPOSURE ON *CELLEPORELLA HYALINA* AND *ELECTRA PILOSA*

2.1. INTRODUCTION

Copper is an essential element to life, being an integral component for a number of enzymes including cytochrome oxidase, which is the terminal electron acceptor for the mitochondrial respiratory chain (Brunori *et al.*, 1981; Green *et al.*, 1984). However, essential elements have the potential to be toxic if levels rise above certain taxa-dependent thresholds (Eichenberger, 1986). Elevated concentrations of copper can impair biological function in a number of ways, either through the formation of oxyradicals, which cause peroxidation of lipid membranes (Viarengo & Nicotera, 1991), or by competition for active sites on proteins, resulting in metal substitutions and consequent structural changes that impair functionality, particularly in enzymes (Eichenberger, 1986). The toxicity of copper has led to its extensive use as a marine antifouling compound (Davenport & Redpath, 1984). In the natural environment, essential elements such as copper are relatively scarce and may be growth-limiting (Rainbow, 1993). However, anthropogenic activities may induce a rise in copper levels where detrimental effects occur, defined as pollution. Copper pollution is usually associated with mining activities and heavy industry. Sedimentary copper levels in excess of 3500 μg Cu g^{-1} have been recorded in some estuaries close to copper mines (Grant *et al.*, 1989). In these areas sudden mass mortalities of shellfish, attributed to copper contamination, have been recorded (Goldberg, 1992). In order to enable adequate pollution control, determination of the effects of prolonged copper exposure and monitoring of the amount of biologically available contaminant are necessary.

This investigation aims to obtain information on the ecologically relevant effects of copper at sublethal concentrations on two species of bryozoan, *Celleporella hyalina* and *Electra pilosa*. This information may not only allow Bryozoa to be used as indicators of seawater quality but may also shed light on the role of genotype on

phenotypic response to low-level chemical stress. Previous work involving the use of Bryozoa in relation to pollution, and more specifically, heavy metal contamination has been relatively limited. Soule and Soule (1981) found no evidence for bioaccumulation of copper in the tissues of seven species of marine Bryozoa. Similar results were found for freshwater Bryozoa from the River Meuse (Belgium)(Henry *et al.*, 1989). Work on lethal concentrations of copper has been restricted to either freshwater species or the larvae of marine species (Miller, 1946; Wisely & Blick, 1967; Bushnell, 1974; Pardue & Wood, 1980) . The only work to investigate prolonged exposure to low concentrations of copper was that of Mundy (1981), using the freshwater phylactolaemate *Cristatella mucedo*. Mundy (1981) observed a significant negative effect on growth of *C. mucedo*, over a 10 day period with copper concentrations as low as 2.5 µg Cu litre $^{-1}$.

Increases in the resistance of populations through exposure to heavy metal contamination are well documented (Bryan, 1976; Bradshaw & Hardwick, 1989; Klerks & Levinton,1989). However, some doubt has been cast on whether sufficient distinctions have been made between genetic adaptation and physiological acclimation (Klerks, 1987). There is only limited evidence of the genetic constituents of chemical resistance in marine invertebrates (Klerks, 1987; Baird *et al.*, 1990; Hoffmann & Parsons, 1991). In addition there is a dearth of information on the more specific relationship between genotype and phenotypic response to chronic toxicant exposure (Baird *et al.*, 1990; Baird *et al.*, 1991).

The use of clonal organisms is advocated, not only in the investigation of the role of genotype in phenotypic response, but also in the use of organisms for ecotoxicological testing (Baird, 1992; Lovett Doust *et al.*, 1993; Forbes & Forbes, 1994). The ease with which colonies of Bryozoa can now be cultured and cloned facilitates their use in factorial experiments designed to elucidate genotype-phenotype relationships in different chemical environments (Hunter & Hughes 1991, 1995; Bayer *et al.*, 1994). The genetic constancy provided by clones, excluding somatic mutations, yields greater accuracy in results, and allows the examination of other components of variability in toxicological testing. For example, clones of *Daphnia magna* have provided insight into the variation between tolerance to chronic and acute toxicity within genotypes (Baird *et al.*, 1990). More recently, work with the hydrobiid gastropod *Potamopyrgus antipodarum* has shown that the elimination of

genetic variability does not ensure reduced phenotypic variance in this species (Forbes *et al.*, 1995). Nevertheless, increasing knowledge of ecotoxicological effects related to genetic diversity is essential to formulating accurate predictions of pollution resistance and consequently creating effective pollution legislation (Forbes & Depledge, 1992; Forbes, 1996). The possibility of using Bryozoa as indicators of seawater quality has not been previously investigated. The advent of routine laboratory culture of bryozoan clones, facilitates their use as potential indicator organisms. Biological assessment of water quality offers great potential for accurately and inexpensively monitoring environmental pollutant levels, which can be greatly dependent on the local physical, chemical and biotic conditions (Lewis & Cave 1982).

Firstly, it is necessary to define four terms used in the context of biological pollution assessment over which there may be some confusion (based on Rainbow, 1995).

1) **Biological monitors** - organisms which denote a degree of ecological change in response to pollution ie. a change in growth or reproduction.
2) **Biomonitors** - organisms which bioaccumulate substances in their tissues.
3) **Bioindicators** - organisms which denote an ecological effect by being present or absent.
4) **Biological indicators** - organisms which can exhibit any or all of the above in response to a toxicant.

Biological water quality assessment can either be laboratory-based or *in situ*. The former enables the standardisation of variables, as in the case of bioassays, while the latter gives the advantage of continuous recording, logging brief but repetitive events in addition to major ones (Lovett Doust *et al.*, 1993). As a group, Bryozoa exhibit several favourable features which may lead to their usage as biological monitors. Sessile habit and clonal organisation of Bryozoa alleviate some of the problems associated with other potential animal candidates. However, *in situ* investigations using Bryozoa as biological monitors are unlikely to be successful, due to the difficulties in distinguishing between effects caused by toxic stress and those caused by other naturally occurring stresses. Previous studies have shown no evidence of the

accumulation of heavy metals in the tissues of Bryozoa, thus eliminating their potential as biomonitors (Soule and Soule, 1981; Henry *et al.*, 1989). Bryozoa seem most suited for the creation of indices of biological water quality using responses such as 'growth' in laboratory bioassays (Stebbing 1979, 1980b). Laboratory based toxicity studies have in the past concentrated on LC_{50} values, which have been criticised for their lack of ecological relevance (Luoma, 1995). Measurements of growth have been heralded as 'useful integrated measures of organism performance' (Forbes & Forbes, 1994). Variations in growth can be the result of physiological and/or behavioural stress induced differences. Although purely physiological measurements may provide an earlier indication of stress, growth is an easily recordable and ecologically relevant measurement (Koehn & Bayne, 1989). However, it is important to take into account the negative impact reproductive activity may have on growth, and to incorporate observations of this parameter into the experimental protocol.

The two species of bryozoan chosen for this study, *C. hyalina* and *E. pilosa*, are both commonly occurring and widely distributed within the temperate intertidal zone (Marcus, 1938; Ryland & Hayward, 1977; Hayward & Ryland, 1979). In these bryozoans, growth occurs by the formation of a laminar sheet of feeding autozooids, which, in turn, provides a permanent and easily observable record of growth. *C. hyalina* also has the advantage of possessing separate male and female zooids which allows sexual partitioning within the colony to be monitored, as well as, number of embryos within ovicells. Furthermore, laboratory culturing methods for these two species have been clearly established (Hunter, 1991; Hunter & Hughes, 1991; Bayer *et al.*, 1994). In this study, the effect of genotype on the phenotypic responses of growth and sexual production is examined in both species in relation to copper exposure. Sexual differentiation in *C. hyalina* also enabled male/female allocation to be examined. Larval survival and capability to metamorphose were also investigated. In addition, the possibility of variation between populations from contrasting habitats was explored in *E. pilosa*.

2.2. MATERIALS AND METHODS

2.2.1. Effect of copper on bryozoan colonies

C. hyalina colonies were established using larvae released from colonies exclusively growing on fronds of *Laminaria saccharina* collected at Church Island, Menai Strait, North Wales (53° 13' N 4° 11' W). *E. pilosa* came from fronds of *Fucus serratus* obtained from Church Island (CI), and also from Hell's Mouth (HM), Abersoch, North Wales (52° 48' N 4° 33' W). Three clones of *C. hyalina* and six clones of *E. pilosa* (3 from CI and 3 from HM) were established on separate glass microscope slides (7.5 x 3.9 cm). Each individual colony was reduced to 20 healthy zooids using a fine mounted needle (0.30 mm x 30 mm). Plastic slide racks holding three clones each were placed in cylindrical polyethylene containers (2 litre coke bottles with the neck section removed). Experimental vessels contained 1 litre of 0.2 µm filtered, UV-irradiated sea water to which was added concentrated copper chloride solution to produce a series of six triplicate treatments 10, 25, 50, 100, 200 and 400 µg Cu l^{-1}, as well as three controls without copper added. The colonies were fed with the cryptophyte *Rhinomonas reticulata* (formerly *Rhodomonas baltica*, Novarino 1992) to achieve a final concentration of ≈100 cells μl^{-1}. Algal cells were kept in suspension via aeration (≈200 ml air min^{-1}) from an airstone at the bottom of each vessel. Containers were emptied and rinsed out every day, fresh sea water, copper chloride and algal suspension were then added. Colonies were also randomised within treatments daily in order to eliminate any container effect. Slides and colonies were cleaned with a fine nylon artists brush weekly and placed into clean plastic slide racks. Salinity (35 ‰) and pH (7.90) remained constant among treatments. The containers were maintained at 18 ± 1 °C in a constant temperature room throughout the 8 week experimental period. Colonies were removed and drawn using a camera lucida at weekly intervals, drawings were later digitized to obtain measurements of colony area and perimeter. Fortnightly counts of sexual zooids were undertaken for the *C. hyalina* colonies only, using a Wild dissecting microscope. Number of sexually mature zooids in colonies of *E. pilosa* were counted only once, at the end of the experiment, due to the difficulty in performing quick and accurate

counts. Counts were made using a Leitz Diavert inverted microscope. Total number of zooids per colony was determined for both species at the end of the experiment.

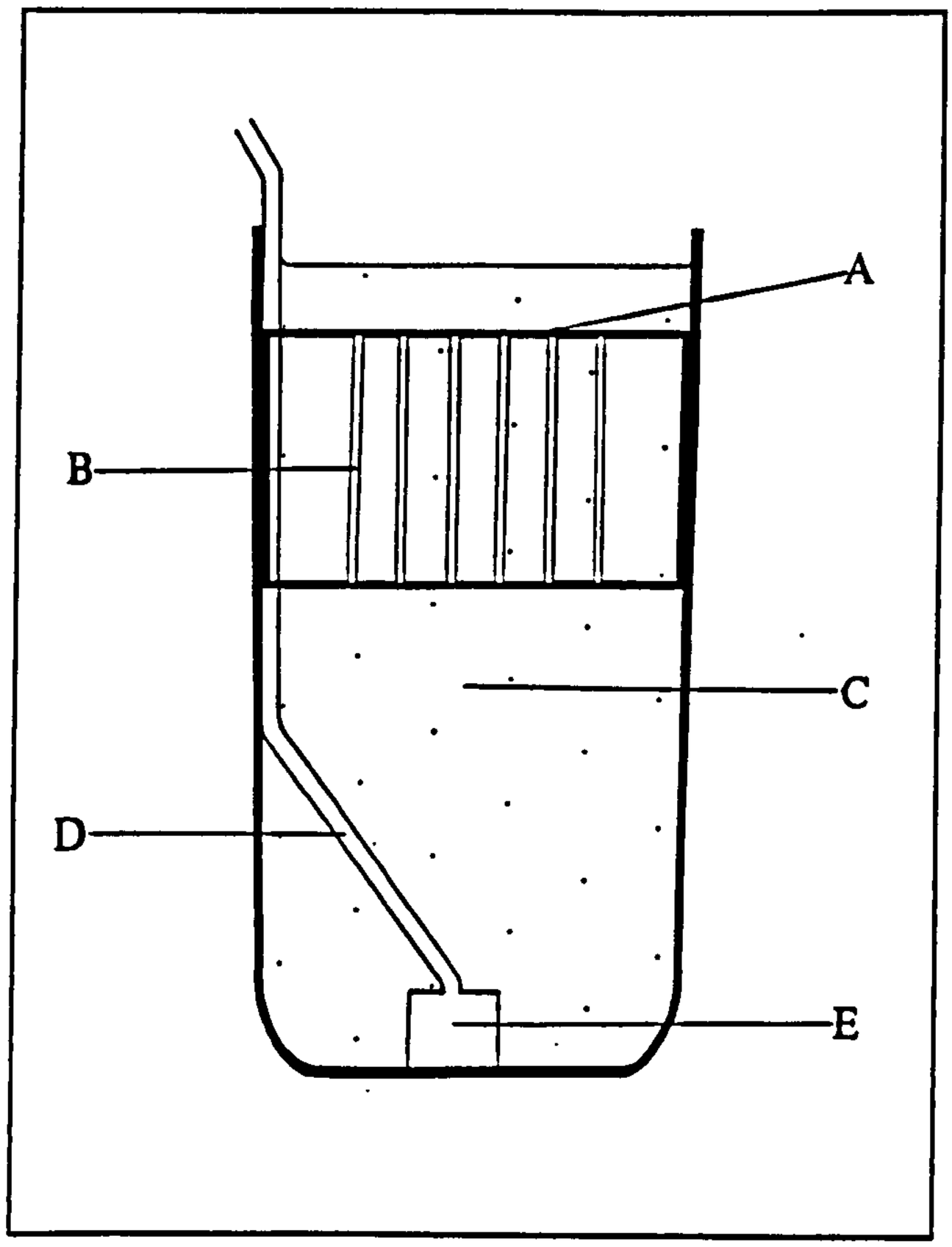


Figure 2.1 Diagram of apparatus used in copper toxicity experiment.

A = Plastic slide rack

B = Glass slides supporting bryozoan colonies

C = Sea water and algal suspension

D = Air supply

E = Airstone

2.2.2. Larval settlement

In the final week of the experiment the effect of copper contamination on larval settlement was assessed. All previously settled offspring were removed from the glass microscope slides also supporting mature colonies. The slides were then returned to the experimental regime for the final seven days of the experiment. At the end of this period all settled larvae were counted using a Wild dissecting microscope. Larval competence, defined as the ability to metamorphose and begin feeding, was also checked once the final measurements of the adult colonies had been taken. Parental colonies and fully metamorphosed larvae were removed from the

experimental glass slides using a scalpel, leaving only recently settled larvae. The remaining larvae were then counted. The slides were returned to the culture vessels containing 0.2 µm filtered sea water and a diet of *R. reticulata* at ≈100 cells μl^{-1}. Sea water and the algal diet were changed daily. Larvae were maintained in this culture regime for seven days, after which time the slides were removed and the number of larvae which had successfully produced feeding zooids were counted using a Wild dissecting microscope.

2.2.3. ICP OES analysis

Water samples were taken for Inductively Coupled Plasma Optical Emission Spectroscopic (ICP OES) analysis, to verify experimental copper levels over time and between particulate and dissolved fractions. Two 5 ml water samples, one filtered to 0.2 µm and the other unfiltered, were collected at the beginning and end of a 24 hour period, before the regular renewal of the culture medium. All samples were frozen and kept at -80°C until ICP OES analysis could be performed.

Frozen samples were thawed and acidified with 5 M Nitric acid (1:1 v/v) then heated to 85°C for 1 hour prior to analysis. ICP OES analysis was undertaken using a Jobin Yvon 138 Trace scan ICP OES. Liquid samples were nebulized before injection into a plasma beam, created by an inert argon column stimulated by a radiofrequency loop. In the plasma the sample becomes thermally excited to the point of atomic emission. The specific emission spectrum of the sample allows its elemental composition to be determined and the intensity of the emitted radiation quantifies its concentration (Willard *et al.*, 1988; Skoog, 1985).

2.2.4. Statistical analysis

Data from observations of colony size, relative colony perimeter (RCP) (Appendix 1.1) and sexual allocation were subjected to the Anderson-Darling test to assess the normality of the data obtained, then analysed accordingly (Minitab, 1996). General linear model (GLM) was applied unless data were found to be non-normally distributed, in which case the nonparametric Kruskal-Wallis test was applied (Zar, 1984). Data from ICP OES analysis was displayed as graphs of percentage difference.

2.3. RESULTS

2.3.1. *C. hyalina*

Both autozooid and sexual zooid production were found to be significantly dependent upon copper concentration (Figs. 2.2 to 2.7). Production of sexual zooids was also found to be significantly influenced by genotype (Figs. 2.3, 2.5, 2.7) However, there was no significant effect of genotype-dosage interaction on either autozooid, sexual zooid or embryo production (Table 2.1).

Table 2.1. Results of ANOVA (GLM, Minitab 11.2) of zooidal allocation for colonies of *C. hyalina* in relation to copper dosage, after a period of 2 months. Sexual zooids and embryos are considered as a proportion of autozooids within the colony.

	Autozooids		*Females*		*Embryos*		*Frontal males*	
	F	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Dosage	6.44	< 0.001*	7.32	< 0.001*	8.25	< 0.001*	3.79	0.006*
Genotype	2.10	0.137	7.64	0.002*	4.63	0.016*	7.49	0.002*
D+G inter.	0.89	0.548	1.06	0.416	1.24	0.298	1.47	0.191

Data presented are F-values (F), with probabilities (P), 'D+G inter.' Copper dosage-genotype interaction. Dosage (N=6); Genotype (N=3); *, significant values;

The number of embryos per female was found not to be significantly influenced by genotype (GLM. $F_{2,35}$=0.08 P=0.925) or genotype-dosage interaction (GLM. $F_{10,35}$=0.73 P=0.694), but was significantly influenced by dosage (GLM. $F_{5,35}$=3.51 P=0.011) (Fig 2.6).

The proportion of basal males per autozooid between test groups was not normally distributed. Therefore, data were analysed using the Kruskal-Wallis test. Copper dosage was found to have a significant effect (H=20.61, df=5, P=0.001) upon the proportion of basal males, but there was no significant effect of genotype (H=1.68, df=2, P=0.432).

Colonies showed a marked increase in autozooid production at copper concentrations of 25 μg l^{-1} and 50 μg l^{-1}, which coincided with an observed increase in females, embryos and frontal males per autozooid (Figs. 2.2 to 2.5). However, the number of embryos per female showed little fluctuation over the range of copper

concentrations except at 200 µg Cu l^{-1} where numbers decreased (Fig. 2.6). The number of basal males per autozooid was lowest at 25 µg Cu l^{-1}, but increased to over double that of controls at concentrations of 200 µg Cu l^{-1} (Fig. 2.7). Also observed at 200 µg Cu l^{-1} was a marked tendency for functional autozooids to be absent from non-peripheral areas of the colony (Fig. 2.8). Relative Colony Perimeter (RCP) (Appendix 1.1) was used to calculate a dimensionless index of colony form (Jebram & Rummert, 1978; Bayer *et al.*, 1994). There was no significant effect of genotype on RCP (H=4.96, df=2, P=0.096). However, dosage was found to have a significant effect on RCP (H=13.95, df=5, P=0.016)(Fig. 2.9). Mortality of colonies was observed at copper concentrations of 400 µg Cu l^{-1} within 14 days. Fifty percent of colonies were dead following a 21 day exposure and no living colonies remained after 35 days within the 400 µg Cu l^{-1} treatment. An individual colony was also lost by the second week in the 200 µg Cu l^{-1} treatment.

Relative sexual allocation was calculated following adjustments for colony size effects, by using area as a covariate. Both females and embryos were unaffected by relative size GLM. $F_{1,34}$=0.02 P=0.896 and GLM. $F_{1,34}$=0.16 P=0.692, respectively. However, frontal males approached near significance with respect to colony size (GLM. $F_{1,34}$=3.94 P=0.055), and basal males were found to be significantly more common in smaller colonies (GLM. $F_{1,34}$=12.72 P=0.001).

The number of settled larvae was significantly affected by copper dosage (H=26.5, df=5, P= < 0.001). Figure 2.10 demonstrates the increase in the numbers of settling larvae at 10 µg Cu l^{-1} and 50µg Cu l^{-1} followed by a decline over the 100µg Cu l^{-1} and 200 µg Cu l^{-1} treatments. The copper concentration at which the highest larval settlement was observed coincides with the highest number of autozooids and ovicells (Figs. 2.2, 2.3 & 2.10). Larval metamorphosis, was not found to be significantly affected by copper dosage (H=3.6, df=5, P=0.604) (Fig. 2.11).

2.3.2. *E. pilosa*

Unfortunately during the first week of the experiment there was an unavoidable total loss of clones from the 200 and 400 µg Cu l^{-1} treatments due to an unidentified contaminant in the batch of filtered seawater used in these containers. Later repetition of the 200 and 400 µg Cu l^{-1} treatments using the same genotypes

revealed mortality in all genotypes within one week of exposure to copper concentrations of 400 µg l^{-1} . At concentrations of 200 µg Cu l^{-1} the mean number of autozooids at the end of the experiment were 450 ± 384 and 345 ± 274 for the Church Island (CI) and Hell's Mouth (HM) populations, respectively. Although direct comparisons between the two sets of data cannot be made, due to the asynchrony of the experiments, the result demonstrates a sharp decrease in growth rate at 200 µg Cu l^{-1} (Fig. 2.12 and 2.13). Origin of colonies proved to be significant with respect to colony growth, colony form (Fig 2.14) and reproductive development in the remaining treatments. For the CI clones, colonial growth was observed to be significantly affected by copper dosage but not by either genotype or the genotype-dosage interaction. Presence of sperm and eggs proved to be significantly associated with copper dosage, as well as genotype for the CI population. Only for zooids containing sperm did genotype-dosage interaction approached near significant values (Table 2.2). A slight increase in growth was detected for the CI clones at 25 µg Cu l^{-1}, the concentration at which the highest proportion of sexual investment was detected (Fig. 2.15). The form of CI colonies was found to be significantly affected only by genotype (H=7.42, df=2, P=0.024).

Table 2.2. General linear model (GLM) analysis of variance for the Church Island (CI) colonies of *E. pilosa*, examining the relationship between copper dosage, genotype and zooidal allocation to sexual and somatic function.

	Autozooids		*Zooids with Sperm*		*Zooids with Eggs*	
	F	*P*	*F*	*P*	*F*	*P*
Dosage	3.90	0.011 *	7.26	<0.001 *	5.85	0.001 *
Genotype	0.28	0.760	6.58	0.004 *	3.65	0.038 *
D+G inter.	0.62	0.755	2.17	0.059	0.79	0.612

Data presented are F-values (F) and probabilities (P). D+G inter. Dosage-genotype interaction term. *, significant values. Dosage (N=6), Genotype (N=3).

The number of autozooids in the HM colonies was significantly related to genotype but not copper dosage. The genotype-dosage interaction effect on autozooid number

also approached statistical significance, probably due to the large clonal variance (Table 2.3).The representatives from this population showed no evidence of an increase in growth related to dosage nor did they produce any zooids containing gametes. Colonies from HM showed significant differences in RCP values between clones (H=13.50, df=2, P=0.001) and between treatments (H=12.96, df=4, P=0.011).

Table 2.3. General linear model (GLM) analysis of variance of the number of autozooids for the HM colonies of *E. pilosa*.

	Autozooids	
	F	*P*
Dosage	1.35	0.274
Genotype	8.83	0.001 *
D+G interaction	2.08	0.070

Data presented are F-values (F) and probabilities (P). D+G interaction, Dosage-genotype interaction; *, significant values. Dosage (N=6), Genotype (N=3).

2.3.3. ICP OES

ICP OES analysis of the water samples showed some variation in levels of copper between filtered and unfiltered samples and over time (Figs. 2.16 and 2.17). Unfortunately, one filtered sample from the 50 µg Cu l^{-1} treatment was lost. The greatest percentage difference over time was 27.86% in the unfiltered control treatment (0 µg Cu l^{-1}) sample. Unexpectedly, a negative value (-5.02%) was recorded in the 100 µg Cu l^{-1} unfiltered sample. This can only be attributed to either sampling error, or release of faecal material containing copper. The greatest difference between the filtered (dissolved) and unfiltered (particulate and dissolved) samples occurred in the control treatment (0 µg Cu l^{-1}) which had a 31.25% difference between the particulate and dissolved copper at time zero. The level of dissolved copper recorded in the control treatment was 3.99 µg Cu l^{-1}, which was within the typical range for Menai Strait seawater (Davenport and Redpath, 1984). Actual copper concentration was generally slightly above that of the allotted copper concentration (Fig. 2.18).

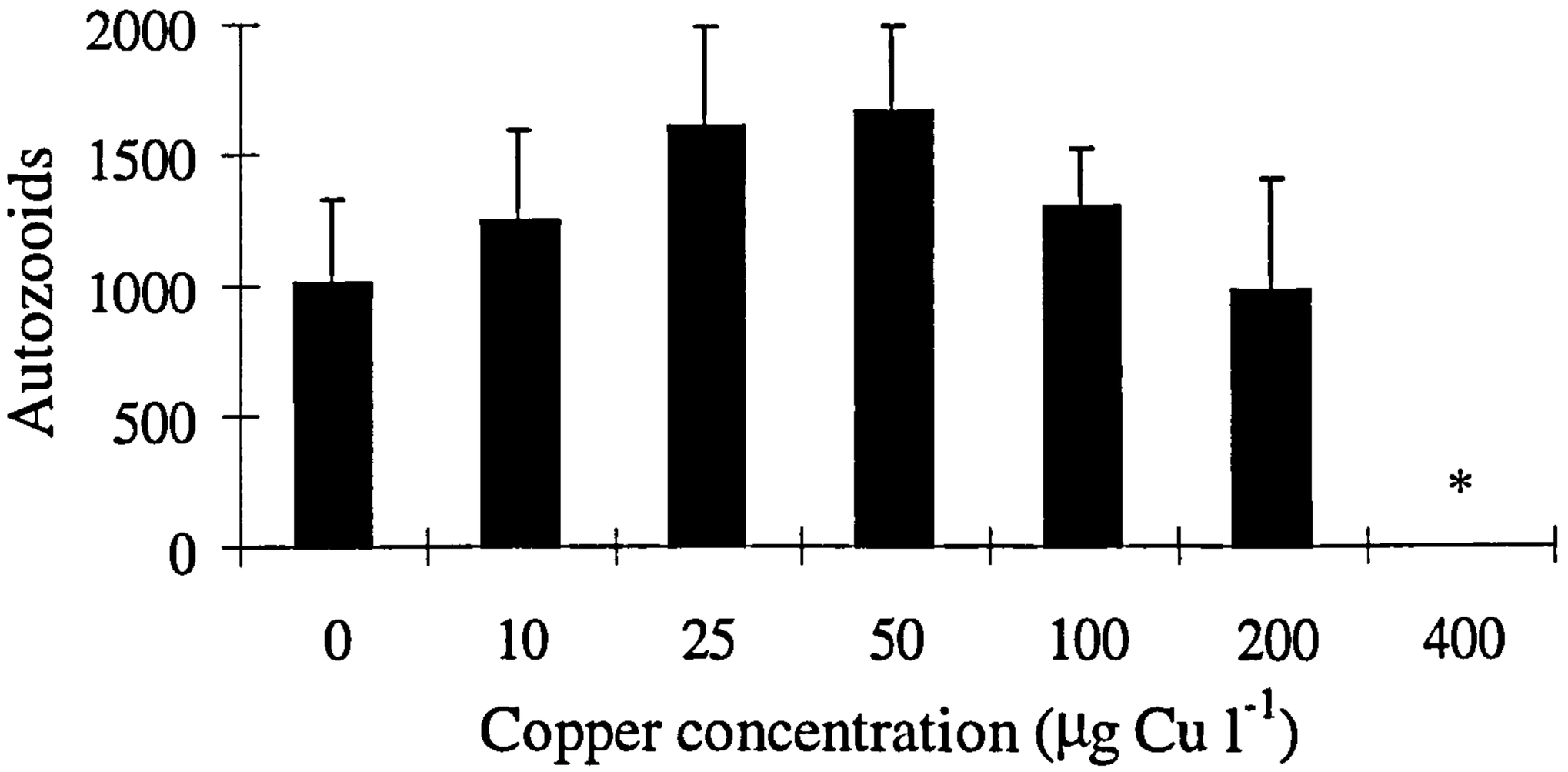


Figure 2.2. Number of *C. hyalina* autozooids (Mean + 1SD) observed over a range of copper concentrations (0, 10, 25, 50, 100, 200, 400 µg Cu l^{-1}) after a 56 day exposure period. *, No surviving colonies. (Colonies n=9, Genotypes n=3).

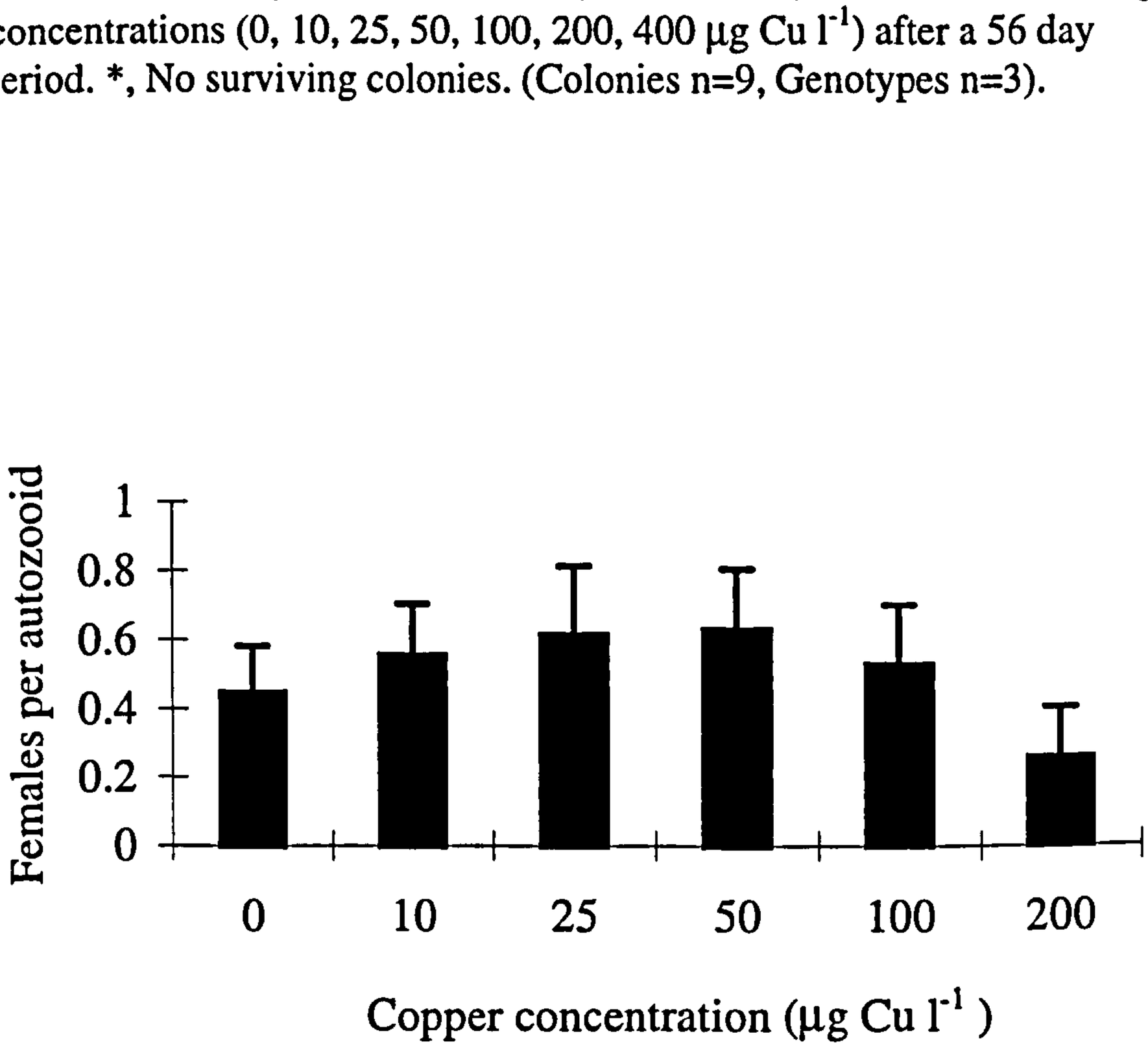


Figure 2.3. Number of *C. hyalina* female zooids (ovicells) per autozooid (Mean + 1 SD) observed over a range of copper concentrations (0, 10, 25, 50, 100, 200 µg Cu l^{-1}) after a 56 day exposure period. (Colonies n=9, Genotypes n=3).

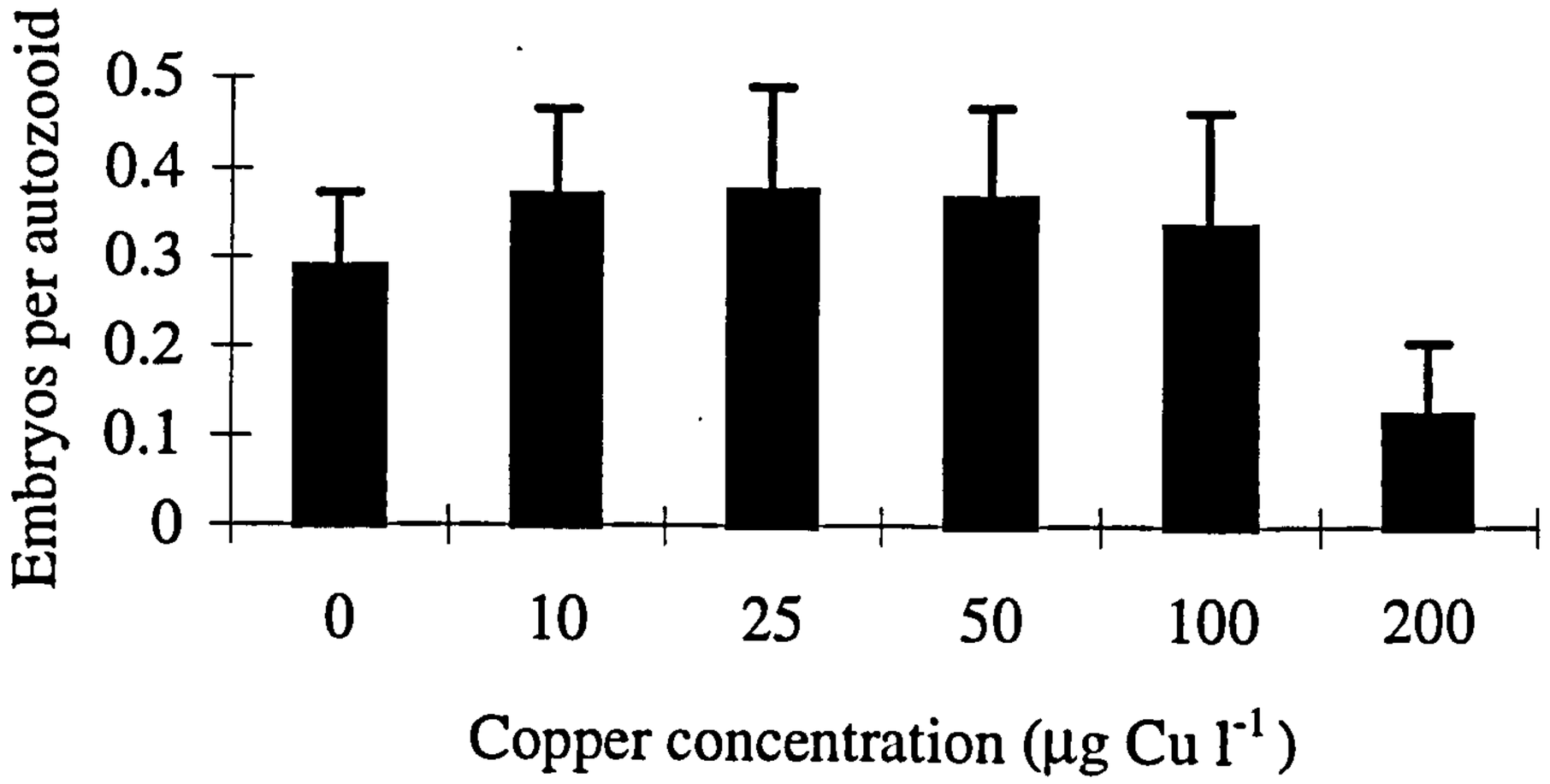


Figure 2.4. Number of *C. hyalina* embryos per autozooid (Mean + 1 SD) observed over a range of copper concentrations (0, 10, 25, 50, 100, 200 µg Cu l^{-1}) after a 56 day exposure period. (Colonies n=9, Genotypes n=3).

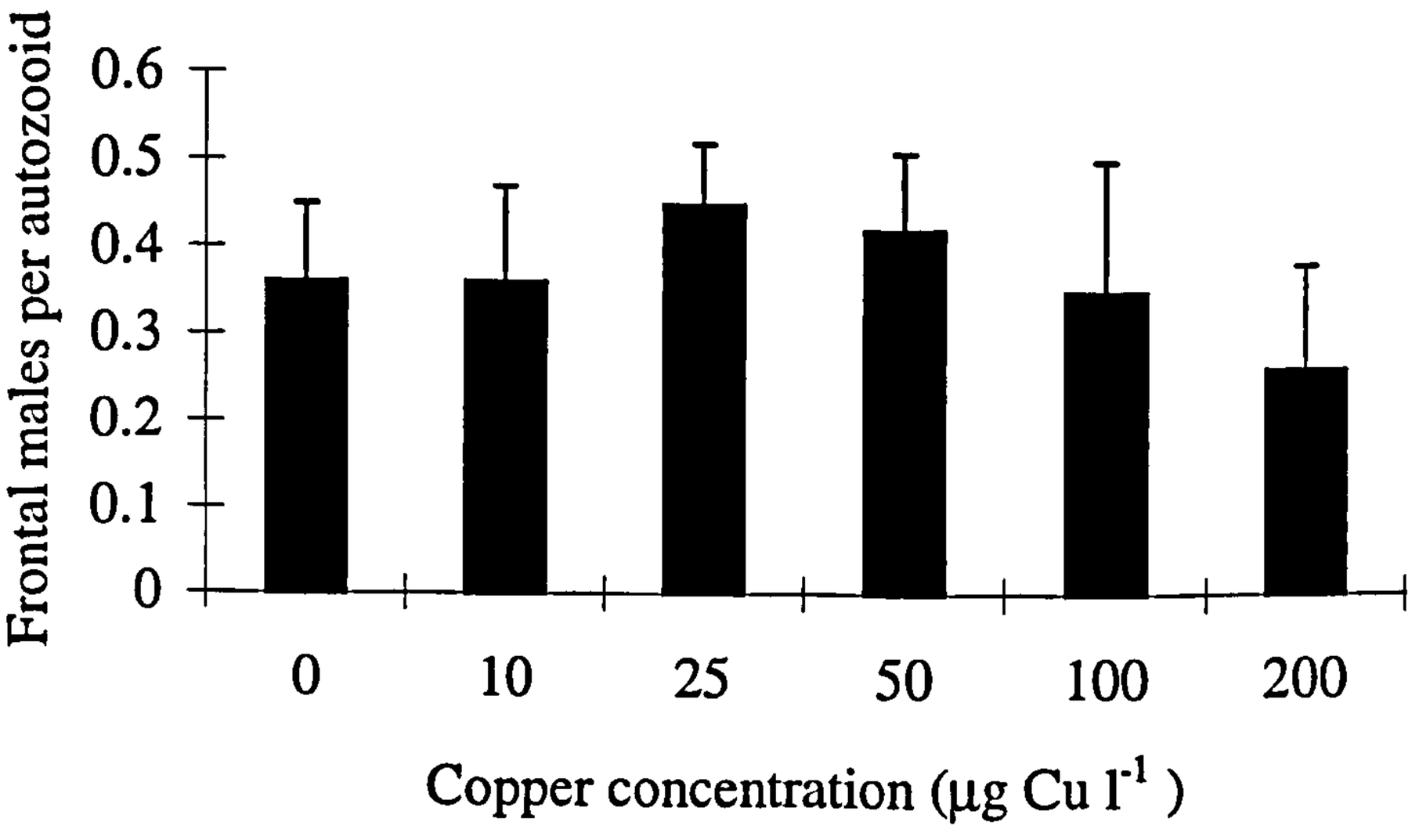


Figure 2.5. Number of *C. hyalina* frontal males per autozooid (Mean + 1 SD) observed over a range of copper concentrations (0, 10, 25, 50, 100, 200 µg Cu l^{-1}) after a 56 day exposure period. (Colonies n=9, Genotypes n=3).

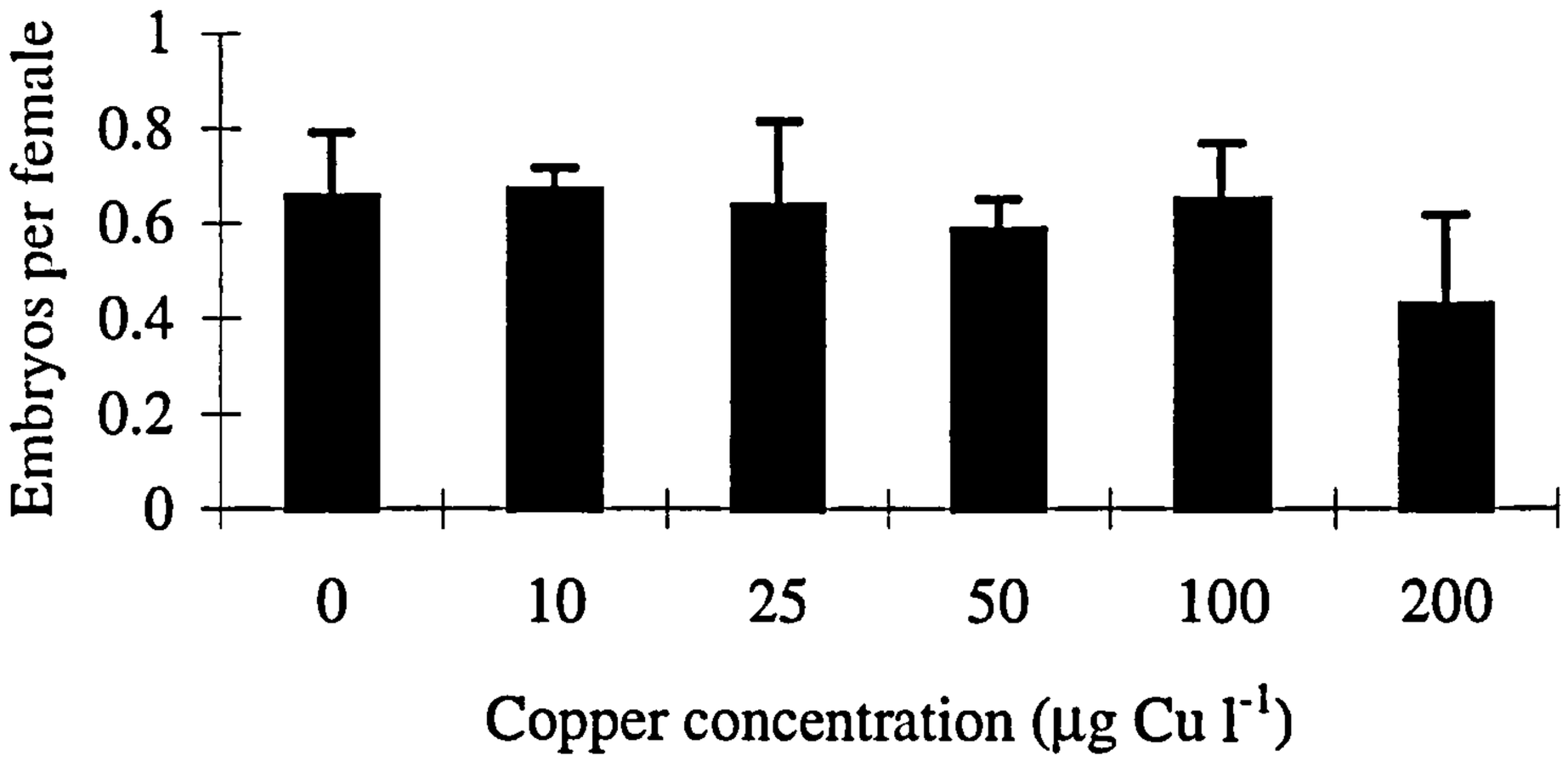


Figure 2.6. Number of *C. hyalina* embryos per female (Mean + 1 SD) observed over a range of copper concentrations (0, 10, 25, 50, 100, 200 µg Cu l^{-1}) after a 56 day exposure period. (Colonies n=9, Genotypes n=3).

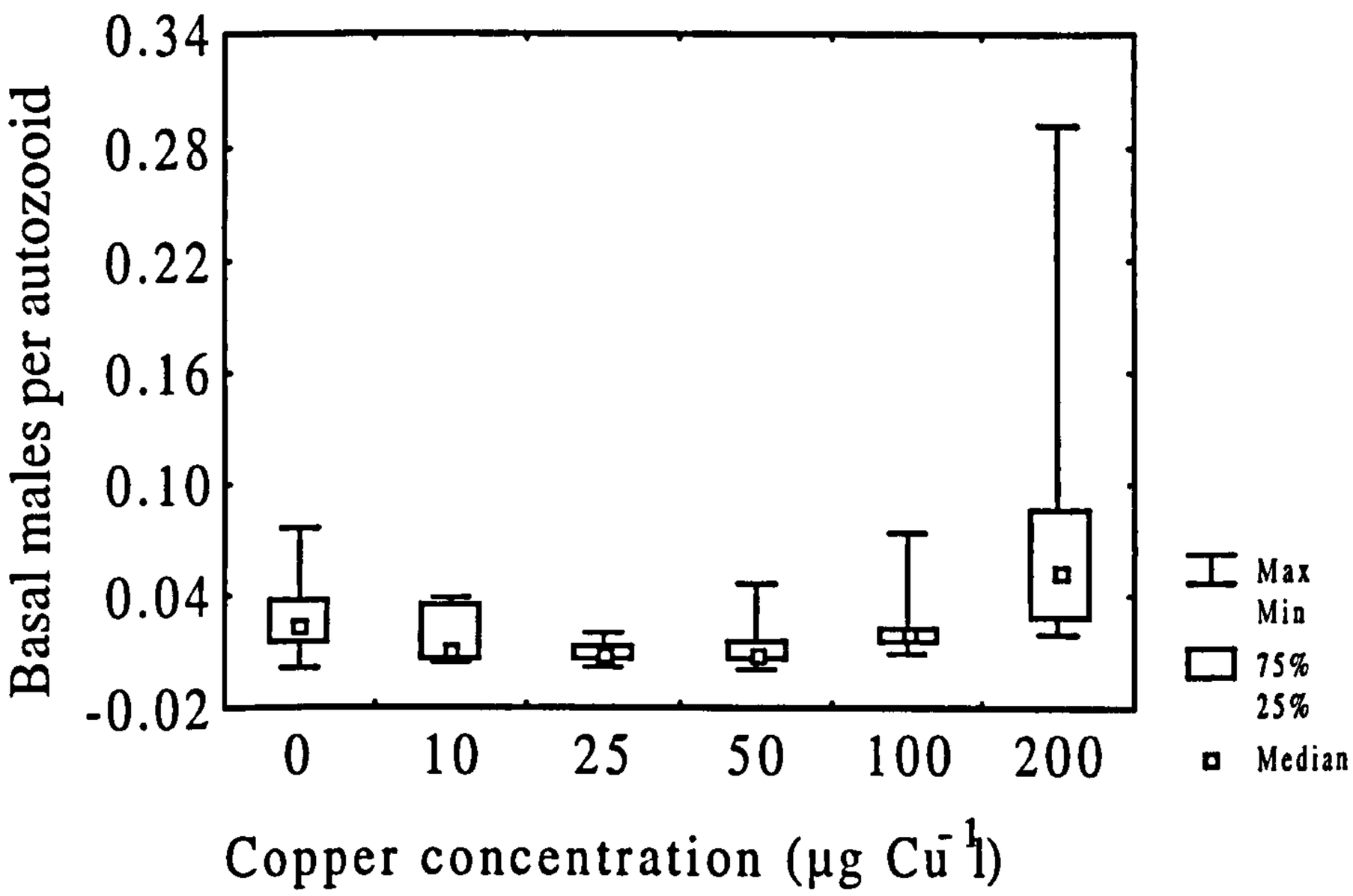


Figure 2.7. Number of *C. hyalina* basal male zooids per autozooid recorded over a range of copper concentrations (0, 10, 25, 50, 100, 200 µg Cu l^{-1}) after a 56 day exposure period. 75% to 25% boxes define upper and lower quartiles. (Colonies n=9, Genotypes n=3).

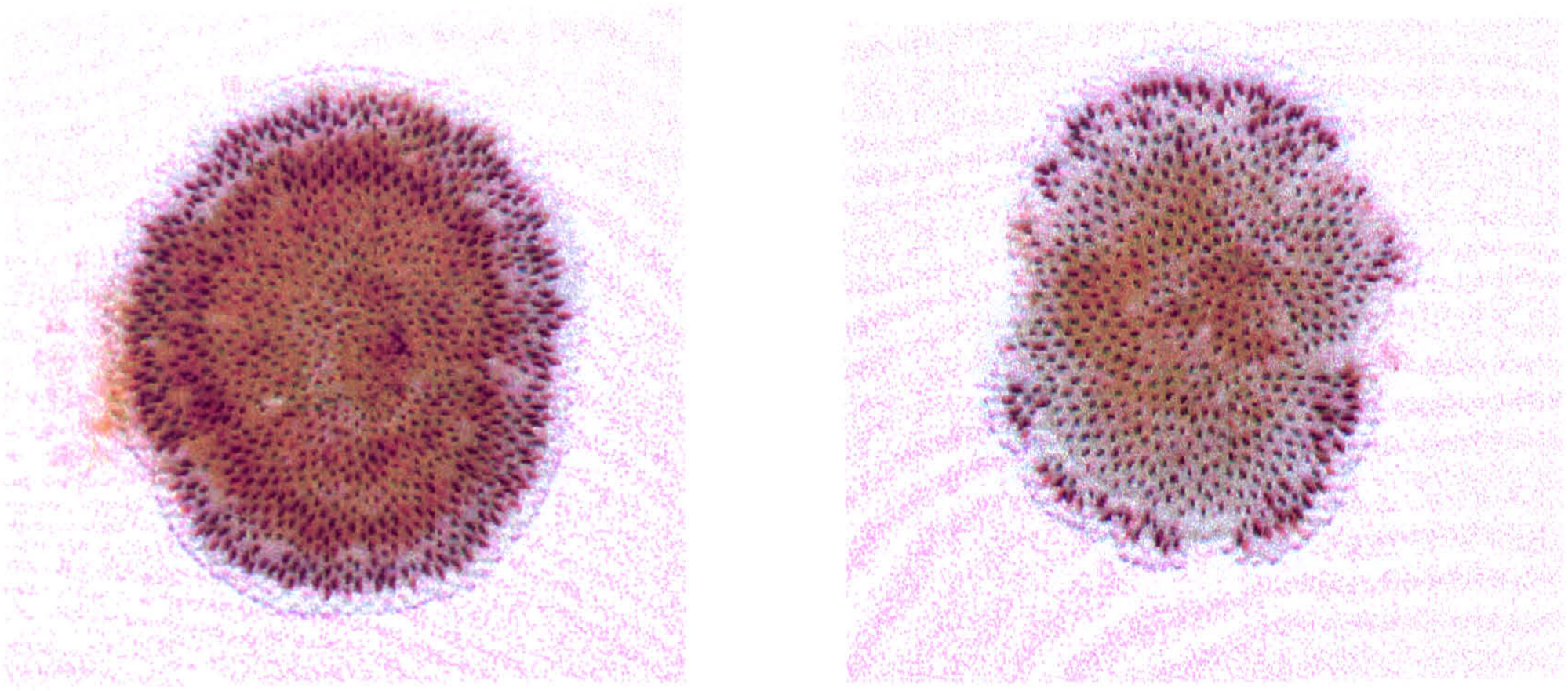


Figure 2.8. Images of two *C. hyalina* clone mates. Feeding zooids indicated by red/brown areas. Clone A1, from treatment with no additional copper (0 µg Cu l^{-1}) added, feeding zooids occur in two concentric rings with a few centrally located. Clone A6, from 200 µg Cu l^{-1} treatment, functional feeding zooids limited to periphery of the colony only.

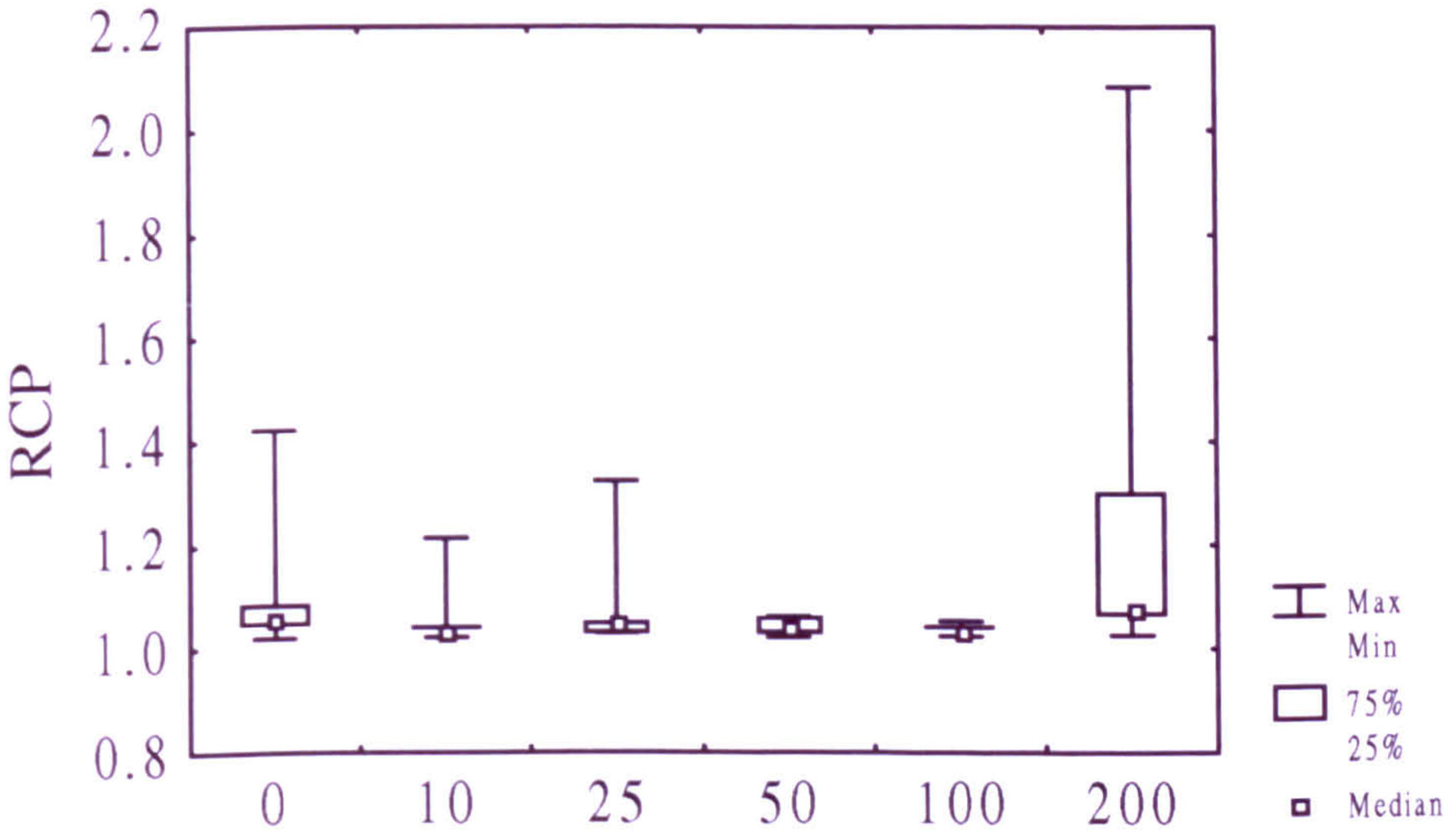


Figure 2.9. Relative Colony Perimeter (RCP) of *C. hyalina* colonies recorded over a range of copper concentrations (0, 10, 25, 50, 100, 200 µg Cu l^{-1}) after a 56 day exposure period. 75% to 25% boxes define upper and lower quartiles. (Colonies n=9, Genotypes n=3).

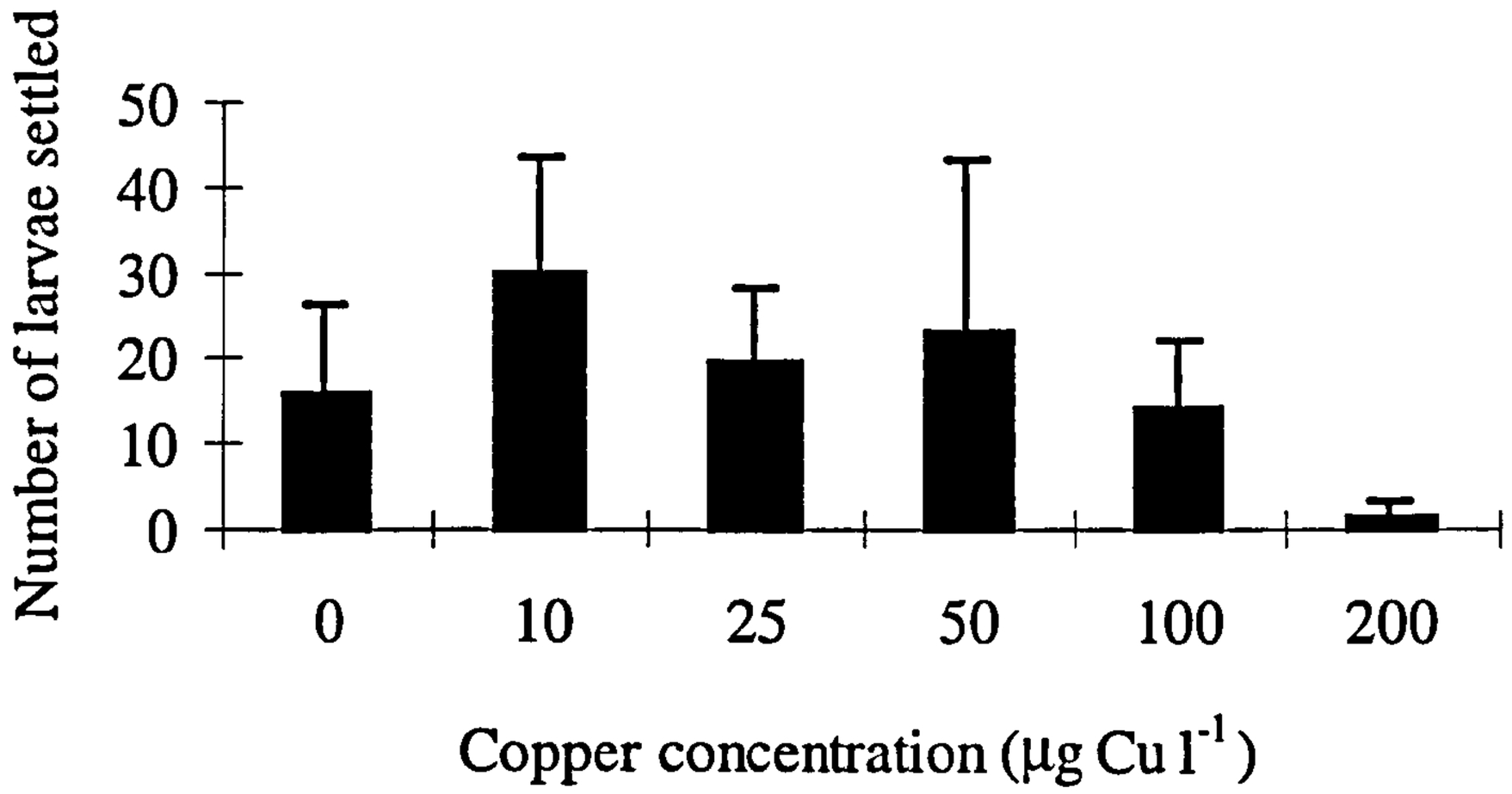


Figure 2.10. Numbers of *C. hyalina* larvae (Mean + 1 SD) settling over the final 7 days of a 56 day exposure period in a range of copper concentrations (0, 10, 25, 50, 100, 200 µg Cu l^{-1}). (n=3).

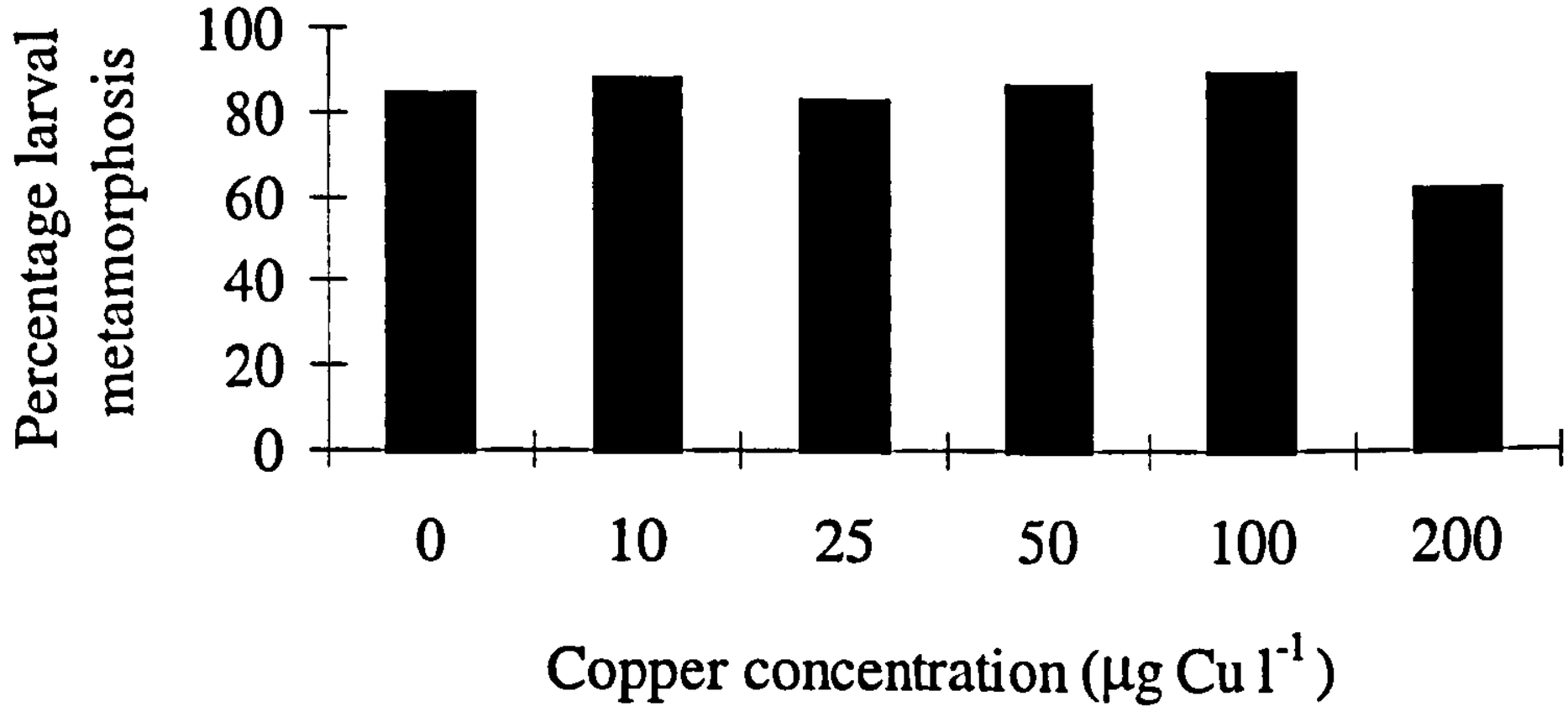


Figure 2.11. Percentage of *C. hyalina* larvae metamorphosing after settlement, when removed from the experimental range of copper concentrations (0, 10, 25, 50, 100, 200 µg Cu l^{-1}) and placed in uncontaminated filtered seawater for 7 days.

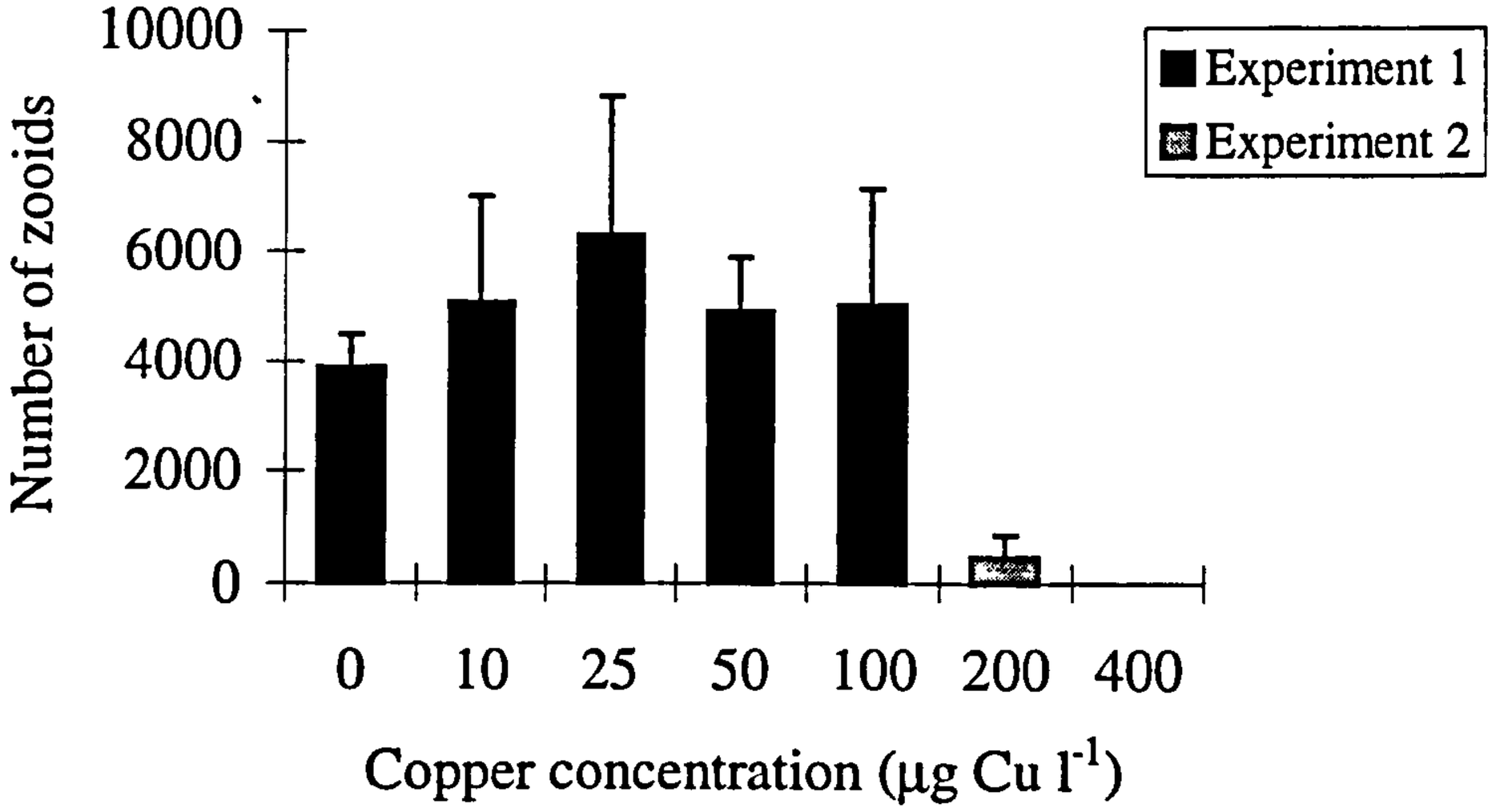


Figure 2.12. Number of CI *E. pilosa* zooids (Mean + 1 SD) observed in experiment 1 over a range of sub-lethal copper concentrations (0, 10, 25, 50, 100 µg Cu l^{-1}) after 56 days of exposure. Data also included for second experiment using only the higher copper concentrations (200, 400 µg Cu l^{-1}). Experiment 2 used the same genotypes as experiment 1 (excluding mutations). (Colonies n=18, Genotypes n=6).

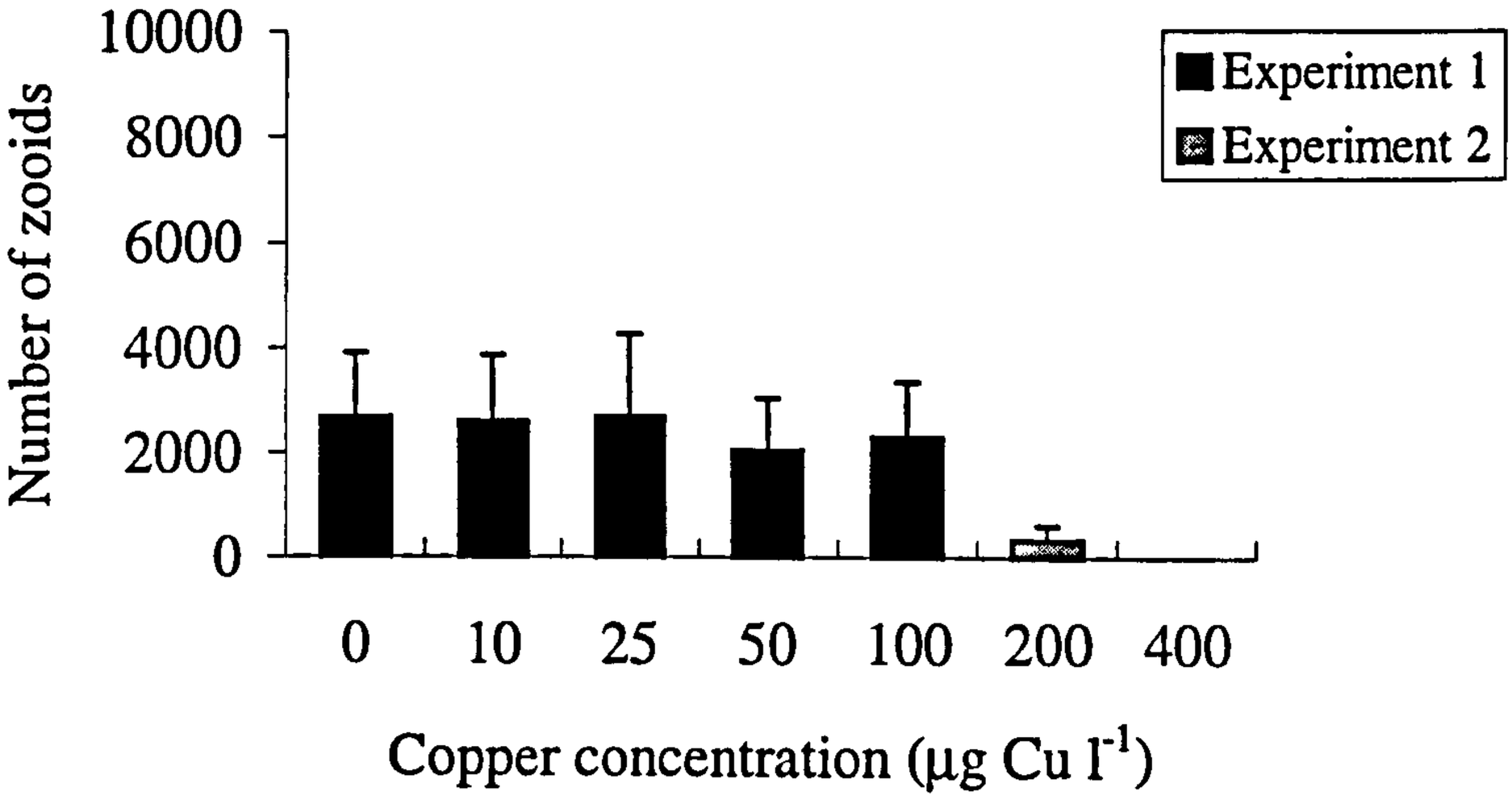


Figure 2.13. Number of HM *E. pilosa* zooids (Mean + 1 SD) observed in experiment 1 over a range of sub-lethal copper concentrations (0, 10, 25, 50, 100 µg Cu l^{-1}) after 56 days of exposure. Data also included for second experiment using only the higher copper concentrations (200, 400 µg Cu l^{-1}). Experiment 2 used the same genotypes as experiment 1 (excluding mutations). (Colonies n=18, Genotypes n=6).

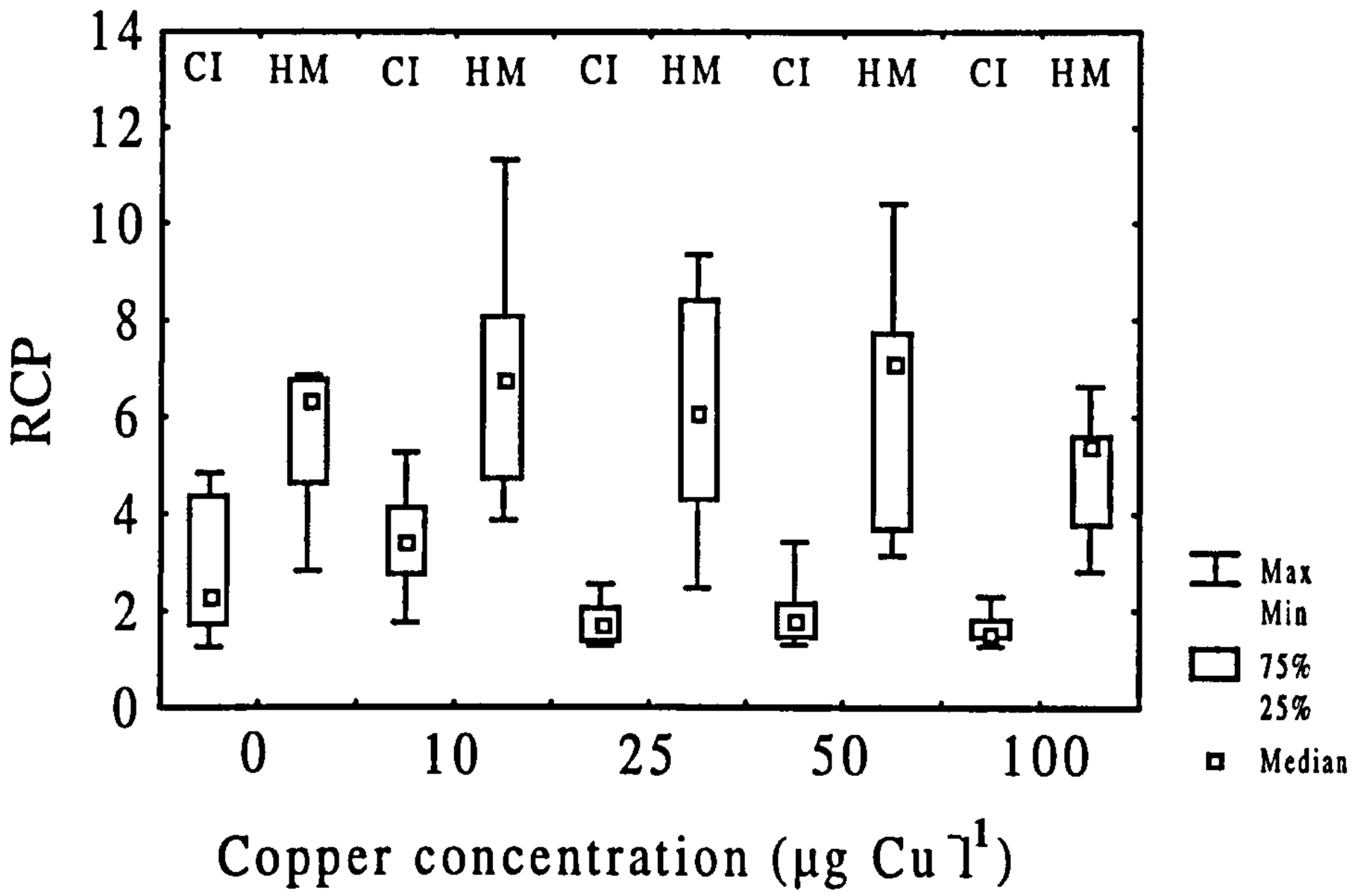


Figure 2.14. Relative Colony Perimeter (RCP) (Mean + 1 SD) of both CI and HM *E. pilosa* colonies, over the range of copper treatments (0, 10, 25, 50, 100 µg Cu l^{-1}) after 56 days of exposure. 75% to 25% boxes define upper and lower quartiles. (Colonies n=9, Genotypes n=3).

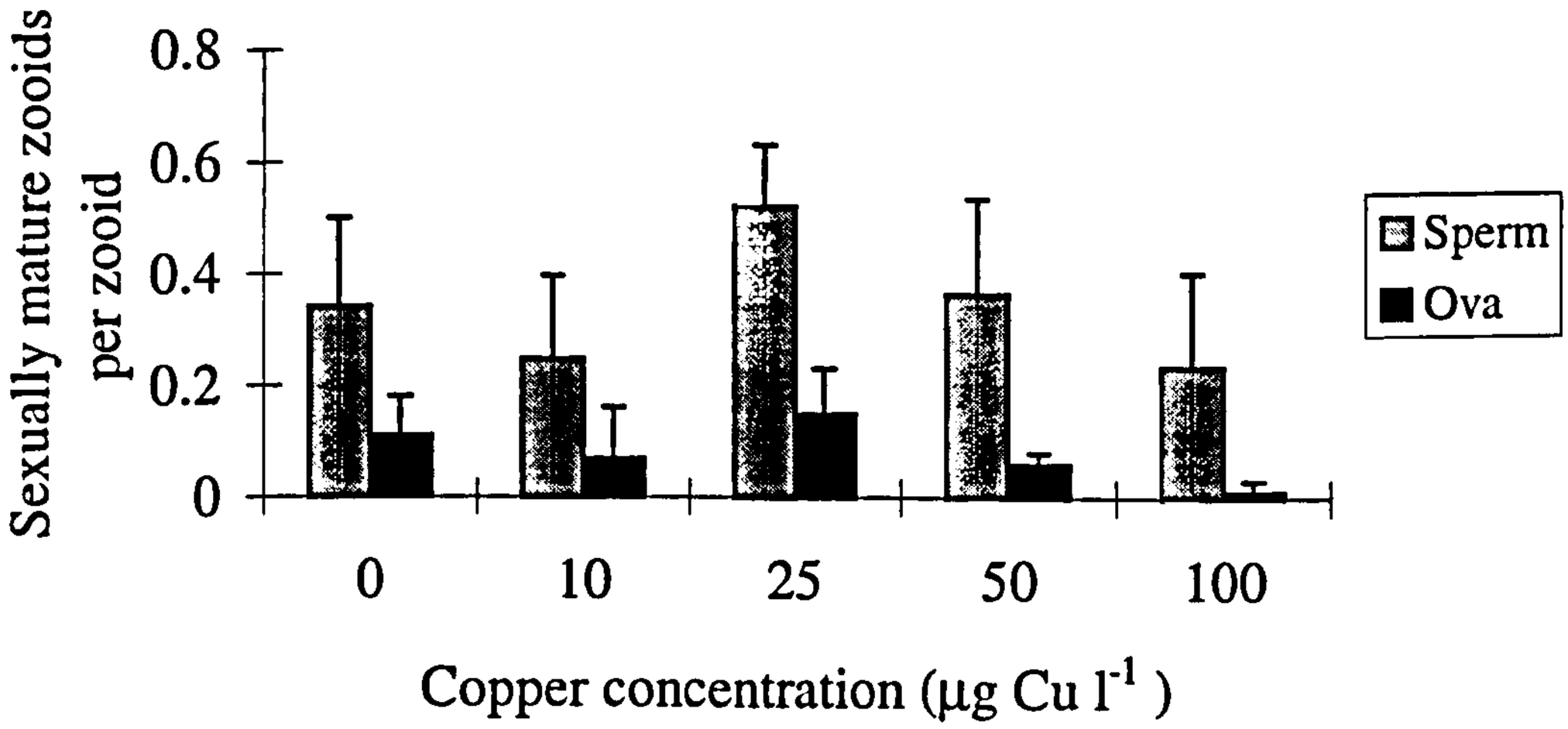


Figure 2.15. Numbers of sexually active zooids per zooid (Mean +1 SD) over a range of copper treatments (0, 10, 25, 50, 100 µg Cu l^{-1}) after 56 days of exposure. Results for CI colonies of *E. pilosa* only, since HM colonies had no sexually mature zooids. (Colonies n=18, Genotypes n=6).

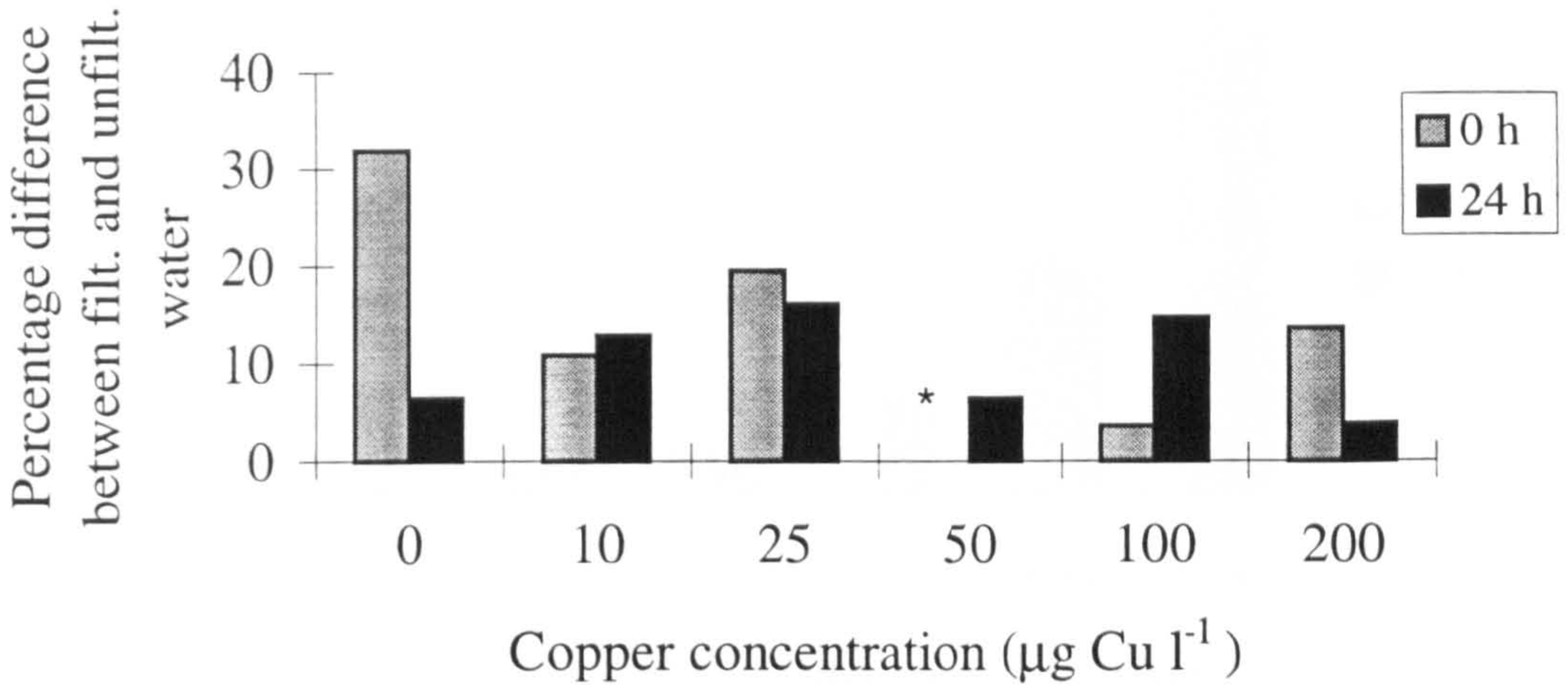


Figure 2.16. ICP OES data, percentage differences between water samples taken at 0 hours and 24 hours, for both filtered and unfiltered samples from the experimental range of copper treatments (0, 10, 25, 50, 100, 200 µg Cu l^{-1}). *, indicates missing data due to lost sample.(n=3).

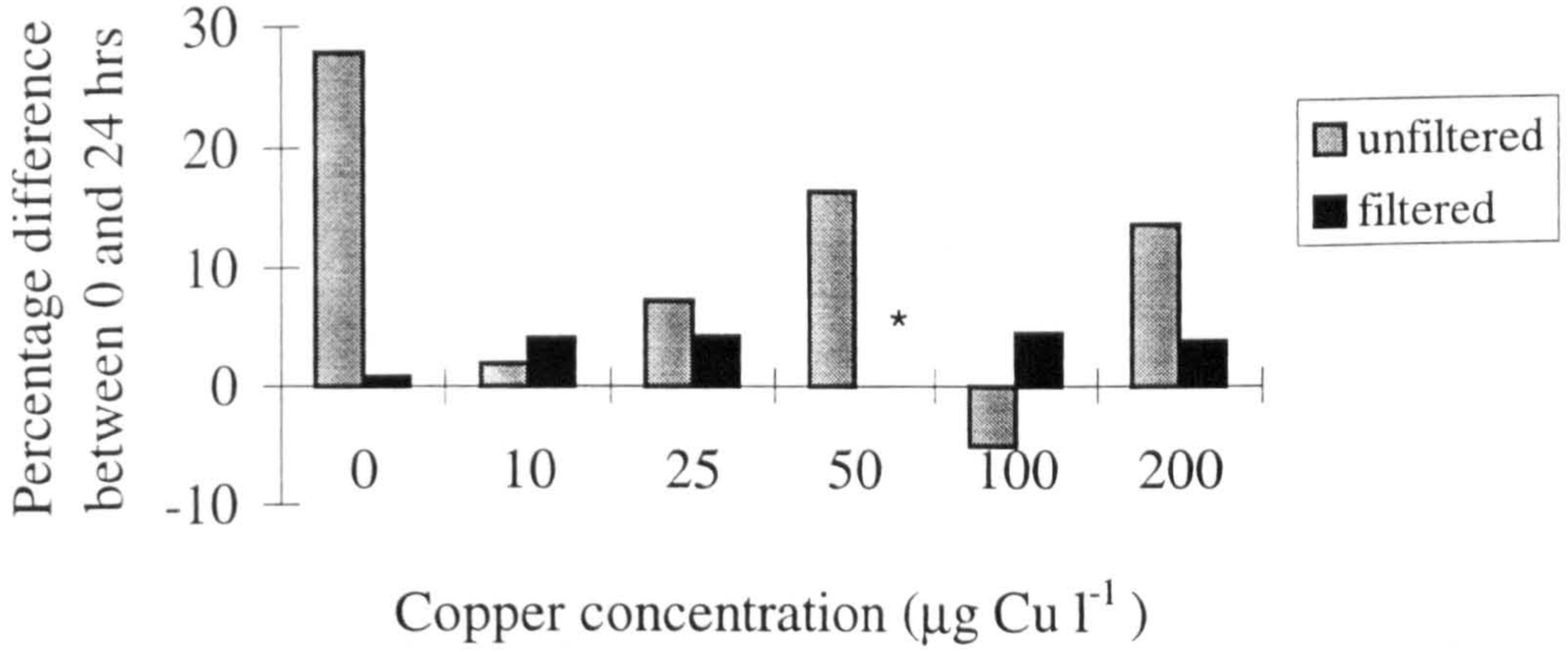


Figure 2.17. ICP OES data, percentage differences between filtered and unfiltered water samples taken from the experimental range of copper treatments (0, 10, 25, 50, 100, 200 µg Cu l^{-1}), at 0 hours and 24 hours later. *, indicates missing data due to lost sample. (n=3).

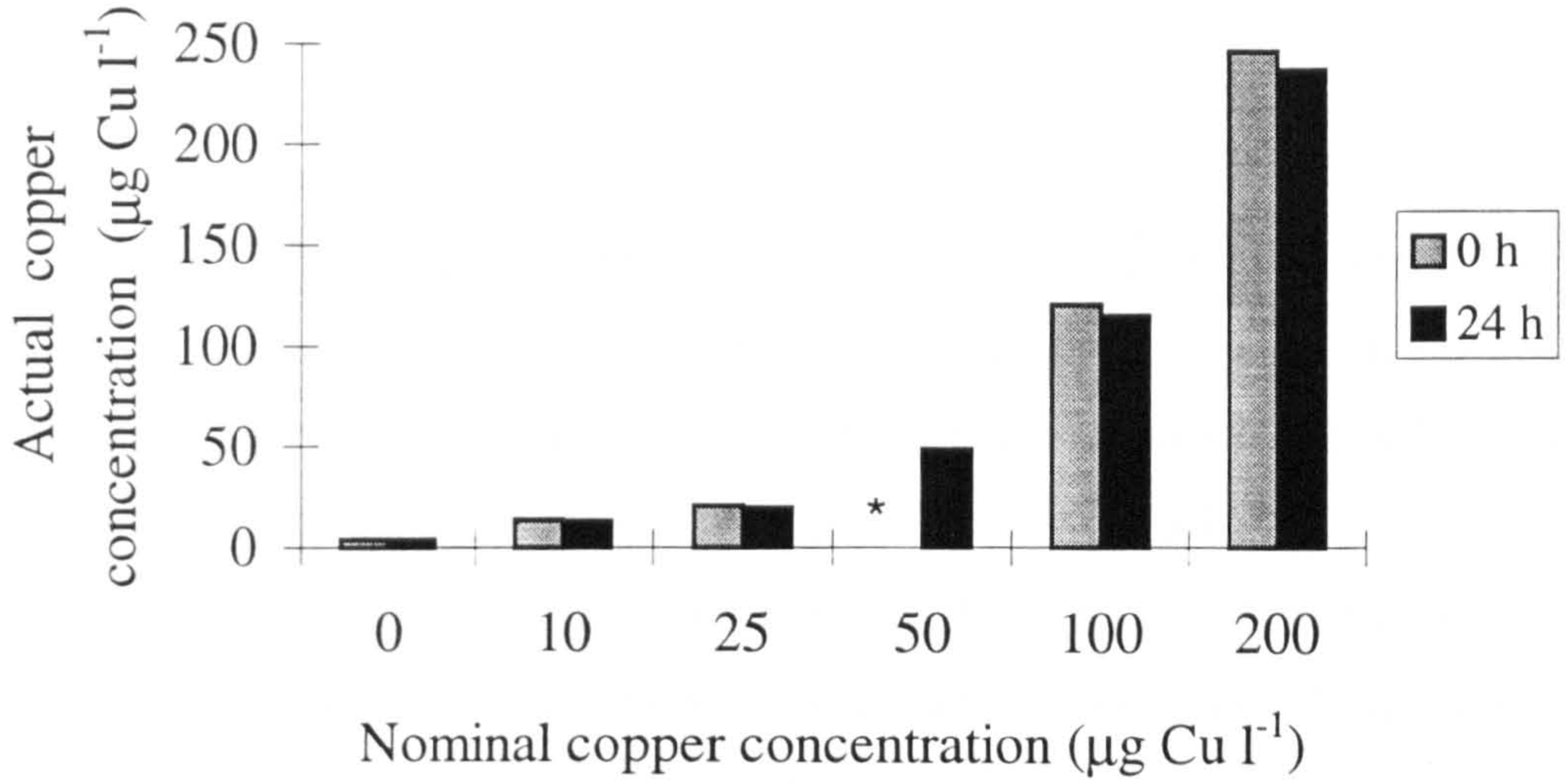


Figure 2.18. ICP OES data, showing variation between allotted and actual copper concentrations in filtered water samples taken from the experimental range of copper treatments (0, 10, 25, 50, 100, 200 µg Cu l^{-1}), at 0 hours and 24 hours later. *, indicates missing data due to lost sample. (n=3).

2.4. DISCUSSION

2.4.1. *C. hyalina*

Although growth of colonies of *C. hyalina* was clearly influenced by both dosage of copper and genotype, no significant genotype-environment interaction was observed. These findings suggest that chronic response to toxicants may be relatively uniform within this population, genotype having relatively little effect on responsiveness. This, in turn, implies that the establishment of clone banks, in order to factor out genotype effects, may not be necessary in this species. Baird *et al.*(1990) demonstrated the large genetic variation between responses in *Daphnia magna* to chronic and acute toxicity, the responses to the former being orders of magnitude less. Evidence also exists supporting the hypothesis that chronic chemical stress resistance has a more complex genetic basis than extreme stress, resulting in a wider but more uniform phenotypic response (Hoffmann & Parsons, 1991). However, the very limited sample of genotypes used in this study means that great caution must be exercised when interpreting the results. Other studies using Bryozoa have demonstrated the importance of genotype in relation to growth rate, sexual allocation and colony form (Hughes & Hughes, 1986b; Bayer *et al.*, 1994; Hunter & Hughes, 1995). Although chronic responses to toxicants may not be heavily influenced by genotype, the factoring out of genetic variation appears to be highly beneficial when considering Bryozoa as biological monitors.

A significant increase in production of frontal male zooids and female ovicells per autozooid was evident in *C.hyalina*, at copper dosages of 25 and 50 µg Cu l^{-1}. It is likely that this rise in production resulted from an overall increase in colony size and a consequent shift in the higher energetic allocation. However, it was at the highest level of copper exposure that the greatest number of basal male zooids per autozooid was produced. The phenomenon of a switch to basal male production during times of stress has been previously observed in colonies maintained by low food concentrations over extended periods of time (Hunter & Hughes, 1993b; P. Manríquez pers. comm.). This shift in production may well increase the likelihood of the perpetuation of the parental genes, by producing large numbers of relatively physiologically inexpensive gametes prior to the demise of the colony. Increases in the allocation to reproductive bodies of stressed hydroids have also been observed

(Stebbing, 1979,1980a; Piraino, 1991). Stebbing (1979) witnessed increases in the gonozooid production of chemically stressed hydroid colonies of up to 24 times that of controls.

In this study, in addition to the observed switch in reproductive effort, colonies exposed to 200 µg Cu l^{-1}, also exhibited a tendency for functional feeding zooids to be peripherally situated. In dosage treatments of 0 to 100 µg Cu l^{-1}, functional feeding zooids occurred in at least two concentric rings within the colony (Fig. 2.8). In contrast, at 200 µg Cu l^{-1} , all zooids behind the peripheral feeding zooids contained brown bodies. Brown body formation has been shown to be related to food ingestion (Bayer *et al.*, 1994), but is also a manifestation of zooid senescence if not followed by polypide regeneration (Bayer & Todd, 1997). The experimental induction of brown body formation through exposure of colonies to stressful environments, such as extremes of temperature and salinity, was first performed by Marcus (1926). It appears that in the 200 µg Cu l^{-1} treatment, polypides have a very limited life span and do not undergo regeneration as in the other experimental treatments, resulting in the occurrence of feeding zooids only at the colony periphery.

The reduction in the number of actively feeding zooids in the 200 µg Cu l^{-1} treatment groups and the increase in basal male production per autozooid, is also accompanied by a decrease in the numbers of embryos per female. This is likely to be the result of decreased energetic acquisition through reduced polypide number and the channeling of available energy into male gamete formation. An increased brooding time of embryos in stressed colonies and a high level of abortion has been observed in other studies (Hughes, 1987; P. Manríquez pers. comm.).

Following exposure to 200 µg Cu l^{-1}, colony form was significantly less circular than in any other treatment. In other encrusting bryozoan species, poor nutrition may produce colonies with a more stellate form, even giving rise to uniserial runners (Winston, 1976; Jebram & Rummert, 1978).

In *C. hyalina*, limited polypide regeneration, increased basal male production, reduced female output and ragged colony form appear to be a good indicators of high organismal stress. In comparison to other marine organisms both species of bryozoan appeared to be extremely resilient to copper exposure (Table 2.4). Two forms of copper can be differentiated based on the assumption that the fraction of copper which passes through a filter of 0.45 µm or less is the 'dissolved' component and the

remainder 'particulate' (Lewis & Cave, 1982). ICP OES analysis enables determination of copper levels in both forms. This method does not, however permit quantification of the species in which the copper exists, which is of considerable importance in toxicological terms. The bioavailability of dissolved copper positively correlates with the activity of the free ion, not the total metal (Sunda *et al.*, 1978; Campbell & Tessier, 1989). The addition of high concentrations of algal cells is likely to result in the release of organic ligands, which by binding with the free copper ions reduce toxicity, making the copper less bioavailable to the bryozoans (Rijstenbil *et al.*, 1994). This may help to explain the apparent resilience of the two bryozoan species to copper contamination in this study. Nevertheless particulate bound copper must not be excluded when considering the overall toxicity of the metal. It has been demonstrated that pollutants can be taken up by organisms from at least some ingested material (Absil *et al*, 1994; James & Kleinow, 1994). Despite this, it seems likely that within the experimental regime of this study, levels of free copper ions were less abundant than in short term mortality studies, which avoid any dietary contribution. However, studies where a dietary component, such as phytoplankton, is introduced, simulate more 'natural' situations. It has been estimated that 65-90 percent of copper is bound by organic complexants and only 1-2 percent exists as free cupric ions in coastal waters (Hart, 1981; Lewis & Cave, 1982).

The possible lack of sensitivity to copper over extended periods make Bryozoa poor candidates for use in water quality bioassays with respect to this element. In addition, previous studies using both atomic absorption analysis of bulk tissue samples and X-ray microanalysis at the cellular level, have shown no bioaccumulation of copper in bryozoan tissues (Soule and Soule, 1981; Henry *et al.*, 1989). However, Baird *et al.*, (1991) demonstrated that even identical genotypes can respond with a much higher sensitivity to one element rather than another, leaving open the possibility that Bryozoa could still prove useful in bioassays for other compounds.

Table 2.4. LT_{50} and LC_{50} values for a variety of marine organisms, after Lewis & Cave (1982).

Phylum	Organism	Cu Conc. µg l^{-1}	N°. Days	Reference
Annelida	*Nephthys hombergi*	250	4	A*
	Nereis diversicolor	540	4	
Cnidaria	*Campanularia flexuosa*	50	<2	B
	Phialidium sp.	36	4	C*
Rotifera	*Brachionus plicatilis*	100	1	
Arthropoda	*Acartia tonsa*	104-311	1	
	Calanus plumchrus	2778	1	
	Carcinus maenas	109	2	D*
	Crangon crangon	295	2	
Mollusca	*Mytilus edulis*	15	28	E
	Tellina tenuis	1000	4	F*
	Haliotis cracherodii	50	4	G*
Bryozoa	*Celleporella hyalina*	400	>21	H
	Electra pilosa	200	>56	

Table 1.4. References. A) Bryan, 1976. B) Stebbing, 1976. C) Reeve *et al.*, 1976*. D) Connor, 1972*. E) Manley, 1980. F) Stirling, 1975*. G) Martin *et al.*, 1977*. H) Present study; *, cited in Lewis & Cave (1982).

Clones of *C. hyalina* exposed to copper dosages of 25 and 50 µg Cu l^{-1} exhibited the highest growth. This increased growth rate may be attributable to either one or a combination of three factors including: 1) an effect of the toxicant on the algal diet, 2) the addition of an essential trace element and 3) hormesis (Stebbing, 1981b). In the first instance, although copper levels reported to inhibit growth of marine phytoplankton cultures are generally greater than 100 µg Cu l^{-1}, structural and physiological effects occur at much lower concentrations (Thomas *et al.*, 1977). Symptoms of chronic copper exposure include reduced cell wall thickness, increased cell size and the release of metal binding ligands (Thomas *et al.*, 1977; Rijstenbil & Wijnholds, 1991; Visviki & Rachlin, 1992, 1994; Rijstenbil *et al.*, 1994; Cid *et al.*,

1997). These changes may increase the digestibility of the cells or, in the case of the released organic ligands, may be absorbed directly (de Burgh & Frankboner, 1979; Oswald, 1986; Best & Thorpe, 1991; Manríquez & Cancino, 1996). In the second instance, the addition of a potentially limiting essential trace metal, such as copper, could also give rise to an increase in growth. The addition of copper has been found to be beneficial to the formation of sea urchin larvae (Wilson & Armstrong, 1961) and growth of barnacle cyprids (Bernard & Lane, 1961). Nonetheless, these advantageous additions of copper were of minute quantities, generally less than 2 µg Cu l^{-1}, far less than the amounts used to initiate a response in this study. Furthermore, Bernard and Lane (1961) found that increasing the dosage to 10 µg Cu l^{-1} resulted in decreased cyprid size and increased mortality. Finally, hormesis, the stimulatory effect of low levels of toxic substances, is a phenomenon which has been found to occur in a variety of unicellular and multicellular organisms (review in Stebbing, 1982). The mechanism of hormesis is still unclear, but it has been suggested that it is associated with an over compensation for inhibitors within the organism's growth control mechanism (Stebbing, 1981a). This may be the first fully documented case of hormesis in bryozoan colonies, although its occurrence was recorded in *Bugula flabellata* larvae by Lynch (1949). Paradoxically, the possibility may arise that where toxic agents such as copper are used in anti-fouling compounds, continuous leaching may give rise to concentrations that eventually promote, rather than inhibit, growth. This may be particularly relevant considering the lack of settling inhibition on copper based anti-fouling products observed for some bryozoan species (Edmonson & Ingram, 1939; Miller, 1946; Weis & Weis, 1992). The settlement of *C. hyalina* larvae was found to be significantly affected by copper dosage but the ability to metamorphose was not. The numbers of larvae settling loosely followed the fluctuations in colony size with dosage, as might be expected. The lack of effect on the ability to metamorphose at relatively high copper concentrations is the more surprising, considering the general sensitivity of larval forms, attributed to greater surface area to volume ratio (Lewis & Cave, 1982). Hoare *et al.*, (1995) subjected *Mytilus edulis* veliger larvae to copper concentrations of 8 µg Cu l^{-1} for approximately 8 weeks in the presence of a microalgal diet including *Rhinomonas reticulata*. The consequent spat survivorship of larvae originating from a population with no previous exposure to copper pollution was significantly reduced. *M. edulis*

veliger larvae are generally considered to be one of the more copper resilient marine larval forms (Lewis & Cave, 1982). This result emphasizes the extremely high level of resilience exhibited by the bryozoan larvae in this study. These findings of unusually high tolerance to copper support previous findings in *Bugula neritina* and *Bugula flabellata* larvae (Lynch, 1949).

2.4.2. *E. pilosa*

The findings of this study provide strong evidence of varied response between the two populations of *E. pilosa*. Individuals from Church Island (CI) exhibited more similar responses to *C. hyalina* from the same site, than did *E. pilosa* from Hell's Mouth (HM). All available evidence suggests that seawater levels of copper do not differ significantly between CI and HM sites (Manley, 1980; Davenport & Redpath, 1984). Variation in local copper concentration can lead to local selection for copper tolerance and may have explained the differences observed in the two populations (Klerks, 1987). The CI clones showed some evidence of an increased growth rate in response to copper exposure at concentrations of 25 μg Cu l^{-1}. Possible explanations for this increased growth are believed to be similar to those previously discussed for *C. hyalina* (this chapter).

Production of sexually active zooids was found to be associated with both genotype and copper dosage. It is likely that the association between copper dosage and reproductive output is directly related to colony size whereby the overall increase in nutritional acquisition from increased number of autozooids provides greater energy for reproductive development. The importance of the genotypic component in reproductive allocation has been documented for *C. hyalina* (Hunter & Hughes, 1995), but has not previously been recorded in *E. pilosa*. Bayer & Todd (1996) found no clear genetic component to sexual maturity in *E. pilosa* and subsequently assumed sexual differences were nominal for the purposes of modeling colony growth. However, in light of the findings of the present study, this assumption may well be in need of revision.

Colony form was significantly affected by genotype in both CI and HM colonies. Only in HM colonies did copper dosage affect colony form. Bayer *et al.* (1994) have documented the significance of genotype effect on colony form in *E. pilosa*. The production of stellate colonies has previously been observed in

encrusting bryozoans exposed to stressful environments, such as poor diet quality (Winston, 1976; Jebram & Rummert, 1978). Although, colonies in such stressful environments became stellate through the formation of distinct uniserial 'runners' of zooids. More recently, the formation of multiserial stellate colonies has been attributed to fast growth in favourable conditions (Okamura, 1992; Bayer & Todd, 1996), stellate colonies being formed due to rapid growth of the primary axis of the colony, with distal and lateral budding taking time to fill in between these areas (Cook, 1977; Silén, 1987; Okamura, 1992). The colonies in this study are observed in the most compact form at exposure levels of 25 µg Cu l^{-1} and to a lesser extent in 50-100 µg Cu l^{-1} exposure level. Therefore, it appears that copper levels within this range either exert insufficient stress levels to induce stellate form, or exert a different type of stress than does a poor food supply. The association between growth rate and form is intertwined with reproductive allocation. Although colonies in the 25 µg Cu l^{-1} treatment initially grew fastest, their per zooid sexual allocation then increased, which, in turn, reduced growth rate and possibly gave rise to a more compact form.

The HM colonies showed no evidence of either hormesis or gonadal development, and a significant difference in zooid number was only observed with respect to genotype. It may be possible that the HM population was heavily stressed by the culture regime utilised in this study (see Chapter 3), which made quantification of responses to further stress difficult. Doubts on the validity of the use of a mono-diet for *E. pilosa* in experimental studies have been expressed by Bayer *et al.* (1994) (see also Chapter 3). However, this result does help to emphasize the degree of caution which should be utilised when considering the responses of different natural populations to low level toxicant contamination.

2.4.3. ICP OES

ICP OES data showed variations in levels of dissolved and particulate bound copper, the largest percentage differences occurring in the control treatment, with no copper added. This can be accounted for by the presence of algal cells in suspension with individual cells possessing their own discrete copper content. Over time a proportion of these cells fall out of suspension or are ingested, consequently reducing the amount of detectable particulate copper. There is, however, no specific trend in

differences between particulate and dissolved copper or temporal differences between the two forms of copper with time. This is probably due to fluctuations in binding and precipitation of dissolved copper, egestion of faecal material, ingestion and settlement of particulate bound copper. Although a degree of variation did occur in copper content, treatment levels remained relatively close to those stipulated, the only possible exception being the control treatment. Copper levels in the control treatment (0 μg Cu l^{-1}) were actually recorded at 3.99 μg Cu l^{-1} for the dissolved copper sample. This result can be attributed to 'naturally' occurring levels of copper in the Menai Strait, from where the experimental seawater was obtained. Previous analysis of seawater from the Menai Strait indicated copper levels to be between 2 and 5 μg Cu l^{-1} (Davenport & Redpath, 1984). The relevance of the results obtained from the ICP OES analysis is limited in respect to the direct toxicity of the experimental environment, due to the inability to distinguish the amount of free copper ions present. Nevertheless, the data obtained verify that the amount of copper in suspension, either particulate or dissolved, did not decrease substantially over the experimental period.

In conclusion, it seems likely that the apparent high levels of resistance of both bryozoan colonies and larvae is due, at least in part, to the rapid binding of free copper ions in the experimental regime by organic ligands. Nevertheless, bryozoan resilience to copper exposure appears to be considerably higher than that observed in other marine species and bears further investigation. The use of Bryozoa as indicators of seawater quality is only likely to be of value in the area of bioassays, where eliminating genetic variation may be highly beneficial. Genotypic effects on sensitivity to chronic copper exposure appear to be limited within populations, but this conclusion may only be loosely implied due to the limited nature of the study. In further studies a greater number of genotypes should be utilised, although this may present logistical difficulties. In addition, a diet that does not bind as readily with the toxicant of interest would be preferable.

CHAPTER 3.

THE INFLUENCE OF FOOD CONCENTRATION ON TENTACLE NUMBER IN *CELLEPORELLA HYALINA* AND *ELECTRA PILOSA*

3.1. INTRODUCTION

Bryozoans are filter-feeding organisms, utilising a crown of ciliated tentacles, the lophophore, which generates a feeding current and captures particles (reviewed in Gordon *et al.*, 1987; McKinney, 1990). The number of tentacles comprising the lophophore is fixed early in ontogeny and remains constant throughout the polypide's life (Thorpe *et al.*, 1986). Each tentacle possesses lateral cilia, which first create a water current to draw particles into the lophophore and then act as a filter (Ryland,1976; Best & Thorpe, 1986a). Most species have additional frontal and latero-frontal cilia, which are thought to create a current directly to the mouth in the lower part of the lophophore and act as sensors to initiate tentacle flicking (Bullivant, 1968; Riisgård & Manríques, 1997). The number and size of tentacles have a substantial influence on the rate at which water may be filtered and, consequently, on the number of particles captured (Riisgård & Manríques, 1997). Best & Thorpe (1983; 1986a; 1994) observed that filter-current velocity rose in response to increasing food concentration and decreasing particle size. More recently, Riisgård & Goldson (1997) have observed that colonies utilise their full filtering capacity until either the zooids become satiated or food concentration drops below a critical level, resulting in a general cessation of feeding. Competition amongst epifaunal assemblages was originally thought to be dominated by spatial acquisition (Stebbing, 1973a, 1973b; Jackson, 1979). However, interactions of feeding currents also appear to play an important role in the formation of competitive hierarchies (Best & Thorpe, 1986b; Okamura, 1985; 1988; 1992). Under the influence of competition for seston, a selective advantage may be conferred by the production of more powerful feeding currents and the production of lophophores with a greater number of tentacles may provide such an advantage (Best & Thorpe, 1986a).

In the past, tentacle number has often been used as a diagnostic feature in bryozoan taxonomy (Hincks, 1880; Prenant & Bobin, 1956). More recently,

however, variation in tentacle number has been attributed to environmental influences, drawing into question the taxonomic value of this character (Jebram, 1973; Thorpe *et al.*, 1986). Thorpe *et al.*, (1986) found that colonies of *E. pilosa* produced a higher number of tentacles per lophophore when in close proximity to conspecifics, but only a slight increase was recorded for competition with non-congenerics. Tentacle number in the genus *Alcyonidium* has received particular scrutiny because of its importance in species classification (Prenant & Bobin, 1956). Cadman & Ryland (1996) recorded no significant variation in tentacle number for colonies of *A. mytili* throughout the year but observed a high degree of variation in laboratory-reared colonies. Recently, Porter *et al.*, (in press) observed a significant increase in tentacle number of *A. hirsutum* colonies with increasing height on the shore. In addition, these authors recorded increases in tentacle number with colony size and proximity to congenerics. However, proximity to non-congenerics appeared to have no effect. The often cited study by Jebram (1973) suggests that faster growth resulted in higher tentacle number within and between colonies, and that food concentration also had a significant effect on tentacle number. However, other than specifying that malnutrition produced a lower number of tentacles in *Triticella elongata,* no quantification of the concentration of algal diet (*Cryptomonas* sp.) was presented to qualify this statement. In a later study, Jebram (1979) also found that tentacle number increased with growth rate of *E. pilosa* but this study also failed to clearly define the various food type concentrations.

The present study aims to investigate the influence of food concentration, food item size and genotype on tentacle number. Two commonly occurring inter-tidal cheilostome bryozoan species have been used, *E. pilosa* and *C. hyalina* . Tentacle number is typically 12 for *C. hyalina* (Hincks, 1880; Hayward & Ryland, 1979) and between 11-15 for *E. pilosa* (Ryland & Hayward, 1977). The advent of routine laboratory culture of these two bryozoans has enabled the implementation of strict experimental regimes which facilitate the examination of the effects of specific variables on colony parameters (Hunter & Hughes, 1991; Bayer *et al.*, 1994).

3.2. MATERIALS AND METHODS

3.2.1. Tentacle number and colony growth

Propagation of experimental colonies of *C. hyalina* and *E. pilosa* is described by Hunter and Hughes (1995) and Bayer *et al.* (1994), respectively. *C. hyalina* colonies were established using larvae released from colonies growing exclusively on fronds of *Laminaria saccharina* collected at Church Island (CI), Menai Strait, North Wales (53° 13' N 4° 11' W). *E. pilosa* came from fronds of *Fucus serratus* obtained from CI, and also from Hell's Mouth (HM), Abersoch, North Wales (52° 48' N 4° 33' W). Three clones of *C. hyalina* and 6 clones of *E. pilosa* (3 from CI and 3 from HM) were established on individual glass microscope slides (7.5 x 3.9 cm). Each ramet was reduced to 20 healthy zooids using a fine mounted needle (0.30 mm x 30 mm). Plastic slide racks, each holding 3 slides supporting colonies, were placed in cylindrical polyethylene containers (2 litre coke bottles with the neck section removed). Each slide held an individual ramet. Experimental vessels contained 1 litre of 0.2 µm filtered, UV-irradiated sea water. Micro-algal suspension was added to the vessels to achieve a series of 3 treatments of differing concentrations of the cryptophyte *Rhinomonas reticulata* (formerly *Rhodomonas baltica*, Novarino 1992) at 50 (R50), 100 (R100) and 200 cells μl^{-1} (R200). An additional treatment containing 100 cells μl^{-1} of *Isochrysis galbana* Parke (Prymnesiophyceae) (Is100) also was prepared. The concentration of algal cells was verified daily using a haemocytometer. Algal cells were kept in suspension by aeration (≈200 ml air min^{-1}) from an airstone at the bottom of each vessel. Containers were emptied daily, rinsed, and fresh sea water and algal suspension were added. Colonies also were randomised within treatments daily in order to eliminate any container effect. Slides and colonies were cleaned with a fine nylon artists' brush weekly and placed into clean plastic slide racks. Salinity (35 ‰) and pH (7.90) remained constant between treatments over time. The containers were maintained at 18 ± 1 °C in a constant temperature room throughout the 8 week experimental period. At the end of this period the colonies were removed and placed in a Petri dish containing filtered sea water and algal suspension. Once the colony began to feed the number of tentacles per lophophore was counted using a Wild dissecting microscope. Each count was repeated 3 times, for each of 20 lophophores from every colony. Lophophores were only counted for

zooids on the colony periphery before the formation of the first brown body. Furthermore, colonies were removed from the treatment vessels and drawn using a camera lucida. Drawings were later digitised to obtain measurements of colony area and perimeter. For the *C. hyalina* colonies, number of autozooids and sexual zooids also were counted, using a Wild dissecting microscope. Total number of zooids and number of sexually mature zooids in colonies of *E. pilosa* were counted using a Leitz Diavert inverted microscope.

3.2.2. Algal settlement

The rate of settlement of algal cells in the experimental vessels was assessed over a 24 hr period for each treatment. Containers were prepared exactly as above except that racks contained only blank glass slides. Number of suspended cells was measured every hour using an electronic particle counter (Elzone model 80 xy fitted with a 76 μm orifice tube). Results were graphically displayed to indicate the algal concentration to which colonies were exposed over time.

3.2.3. Statistical analysis

Data from observations of tentacle number, colony size and sexual allocation were subjected to the Anderson-Darling test to assess the normality of the data obtained, then analysed accordingly (Minitab, 1996). General linear model (GLM) was applied unless data were found to be non-normally distributed, in which case the Kruskal-Wallis test was applied (Zar, 1984). Data for frequencies of numbers of tentacles were analysed using Chi-square analysis.

3.3. RESULTS

3.3.1. *C. hyalina*

Variation in tentacle number was found to be very limited in *C. hyalina*, 85 % of the lophophores possessing 12 tentacles (Table 3.1). No significant difference was detected in tentacle number between the various cell concentration treatments (H=4.46 df=3 P=0.216). A significant difference was observed in the frequency of lophophores deviating from the modal number of tentacles between treatments (Chi-Square =10.00, df = 3, P = <0.05)(Fig. 3.2). This was largely due to the Is100 and R50 treatments having less tentacle number variation than the R100 and R200 treatments (Fig. 3.1). Tentacle number variation was not significantly different between genotypes (H=0.49 df=2 P=0.781)(Fig. 3.3). Colony area was not found to correlate with tentacle number (R^2=8.0 % $F_{1,34}$=2.97 P=0.094)(Fig. 3.4).

Table 3.1. Summary of tentacle number data for colonies of *C. hyalina*.

Treatment	*Mean*	*Mode*	*Median*	*Stdev*	*Range*	*n*
R50	11.806	12	12	0.397	11-12	180
R100	11.806	12	12	0.424	10-12	180
R200	11.822	12	12	0.498	8-12	180
Is100	11.922	12	12	0.265	11-12	180

R. reticulata treatments, R50 (50 cells μl^{-1}), R100 (100 cells μl^{-1}), R200 (200 cells μl^{-1}), *I. galbana*, Is100 (100 cell μl^{-1}). Number of Colonies (n=36).

Autozooid number was significantly affected by treatment, as was the number of frontal males per autozooid and RCP (Relative Colony Perimeter, Appendix 1.1) (Table 3.2) (Figs 3.5, 3.7 & 3.9). Genotype was found to have a statistically significant effect on the number of females per autozooid produced and RCP. Number of basal males per autozooid was found to be significantly different between treatments (H=7.82, df=3, P=0.05) but not between genotypes (H=0.13 df=2 P=0.936). A much higher proportion of basal males occurred in the *R. reticulata* 200 cells μl^{-1} treatment (Fig. 3.8). Genotype had a marked effect on number of frontal males, although this was not statistically significant.

Table 3.2. Results of ANOVA (GLM) of zooidal allocation and RCP for colonies of *C. hyalina* in response to variable food concentration, following a 56 day treatment period. Sexual zooids are considered as a proportion of autozooids within the colony.

	Autozooids		*Females*		*Frontal males*		*RCP*	
	F	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Treatment	11.85	<0.001*	0.39	0.764	7.75	<0.005*	6.71	<0.005*
Genotype	1.69	0.206	7.25	<0.005*	3.27	0.056	4.07	<0.05*
T+G inter.	0.65	0.691	1.07	0.407	0.67	0.678	1.02	0.439

Data presented are F-values (F), with probabilities (P), 'T+G inter.' treatment -genotype interaction. Treatment (n=4), Genotype (n=3); *, significant values.

Regression analysis indicated that the number of females per autozooid (R^2=5.5 % $F_{1,34}$=1.97 P=0.169) and basal males per autozooid (R^2=0.0 % $F_{1,34}$=0.01 P=0.935) were not dependent on colonial area. However, frontal males per autozooid was directly proportional to colonial area (R^2=20.0 % $F_{1,34}$=8.48 P=0.006).

3.3.2. *E. pilosa*

Tentacle number per lophophore varied between 11 and 15 tentacles (Table 3.3). A significant difference was detected in tentacle number between colonies from CI and HM (H=528.8 df=1 P=<0.001) (Fig. 3.10.) Consequently, colonies were analysed separately for each site.

Table 3.3. Summary of tentacle number data for colonies of *E. pilosa* for different treatment groups presented separately for each collection site.

Treatment	*Site*	*Mean*	*Mode*	*Median*	*Stdev.*	*Range*	*n*
R50	HM	13.43	14	14	0.669	11-14	180
	CI	12.24	12	12	0.594	11-14	180
R100	HM	12.55	12	12	0.786	11-15	180
	CI	12.55	12	12	0.786	11-15	180
R200	HM	13.43	14	13	0.892	11-15	180
	CI	12.22	12	12	0.746	11-15	180
Is100	HM	13.22	13	13	0.479	12-14	180
	CI	12.17	12	12	0.596	11-14	180

R. reticulata treatments, R50 (50 cells μl^{-1}), R100 (100 cells μl^{-1}), R200 (200 cells μl^{-1}). *I. galbana*, treatment Is100 (100 cell μl^{-1}).

Significant differences in tentacle number between treatments and between clones were observed in colonies from HM and CI (Table 3.4). Both CI and HM colonies produced the highest number of tentacles per lophophore in the R100 treatment (Fig. 3.10).

Table 3.4. Kruskal-Wallis test for tentacle number variation of *E. pilosa* colonies, between clones and treatments for both collection sites.

Site		*H value*	*df*	*P value*
HM	Treatment	29.22	3	<0.001*
	Clone	43.62	2	<0.001*
CI	Treatment	23.36	3	<0.001*
	Clone	9.99	2	<0.01*

Data presented are H-values (H), degrees of freedom (df) and probabilities (P); *, significant values.

Regression analysis indicated no significant relationship between tentacle number and colonial area between colonies originating from either HM (R^2=1.4% $F_{1,34}$=0.48 P=0.494) or CI (R^2=0.0% $F_{1,34}$=0.01 P=0.943). Zooid number did not differ significantly between sites (H=2.11 df=1 P=0.146). Colonies were found to produce the largest number of zooids in the R100 treatment after the 56 day experimental period (Fig. 3.11.). RCP was statistically different between sites but no significant interaction effect on RCP was found between site and treatment (Table 3.5). No relationship between area and RCP was detected (R^2=0.8% $F_{1,70}$=0.54 P=0.464).

Table 3.5. Results of ANOVA (GLM) for RCP for colonies of *E. pilosa* from sites HM and CI in response to variable food concentration, following a 56 day treatment period.

	F	*df*	*P*
Treatment	24.10	3	<0.001*
Site	5.34	1	<0.005*
T+S Inter.	0.71	3	0.547

Data presented are F-values (F), with probabilities (P), 'T+S inter.' treatment -site interaction; *, significant values.

RCP values for colonies from both HM and CI were significantly different between treatments and clones. A significant treatment-clone interaction effect on RCP was also observed (Tables 3.6 & 3.7). Colonies in the R100 and R200 treatments were generally more circular in form than those in the R50 and Is100 treatments (Tables 3.6 & 3.7)(Fig. 3.12). Overall, HM colonies tended to have higher RCP values than CI colonies (Table 3.5)(Fig. 3.12).

Table 3.6. Results of ANOVA (GLM) for RCP for colonies of *E. pilosa* from HM in response to variable food concentration, following a 56 day treatment period.

	F	*df*	*P*
Treatment	7.74	3	<0.005*
Clone	4.94	2	<0.05 *
T+C Inter.	3.48	6	<0.05 *

Data presented are F-values (F), with probabilities (P); 'T+S inter.' treatment -site interaction; *, significant values.

Table 3.7. Results of ANOVA (GLM) for RCP for colonies of *E. pilosa* from CI in response to variable food concentration, following a 56 day treatment period.

	F	*df*	*P*
Treatment	4.65	3	<0.05 *
Clone	21.32	2	<0.001*
T+C Inter.	8.90	6	<0.001*

Data presented are F-values (F), with probabilities (P), Abbreviation, 'T+S inter.' Is the treatment -site interaction; *, significant values.

The number of colonies producing spermatozoa and ova was found to differ significantly between sites (H=13.81 df=1 P=<0.001)(H=8.05 df=1 P=<0.005) for male and female gamete producing colonies, respectively. HM colonies produced no ova in any of the treatments (Fig 3.14), however, sperm-producing colonies were significantly different in number between treatments (H=25.22 df=3 P=<0.001) with spermatozoa only being produced in the Is100 treatment (Fig 3.13). CI colonies produced ova in all but the Is100 treatment. Number of CI colonies producing

spermatozoa was significantly different between clones (H=10.60 df=2 P=<0.01) and treatments (H=15.57 df=3 P=<0.005) with the most sperm producing colonies being present in the R100 treatment (Fig. 3.13).

3.3.3. Algal settlement

Due to settling out of the algae from suspension, colonies were exposed to a reduction in algal cell concentration in all treatments of approximately 35 % over a 24 h feeding period (Fig. 3.15).

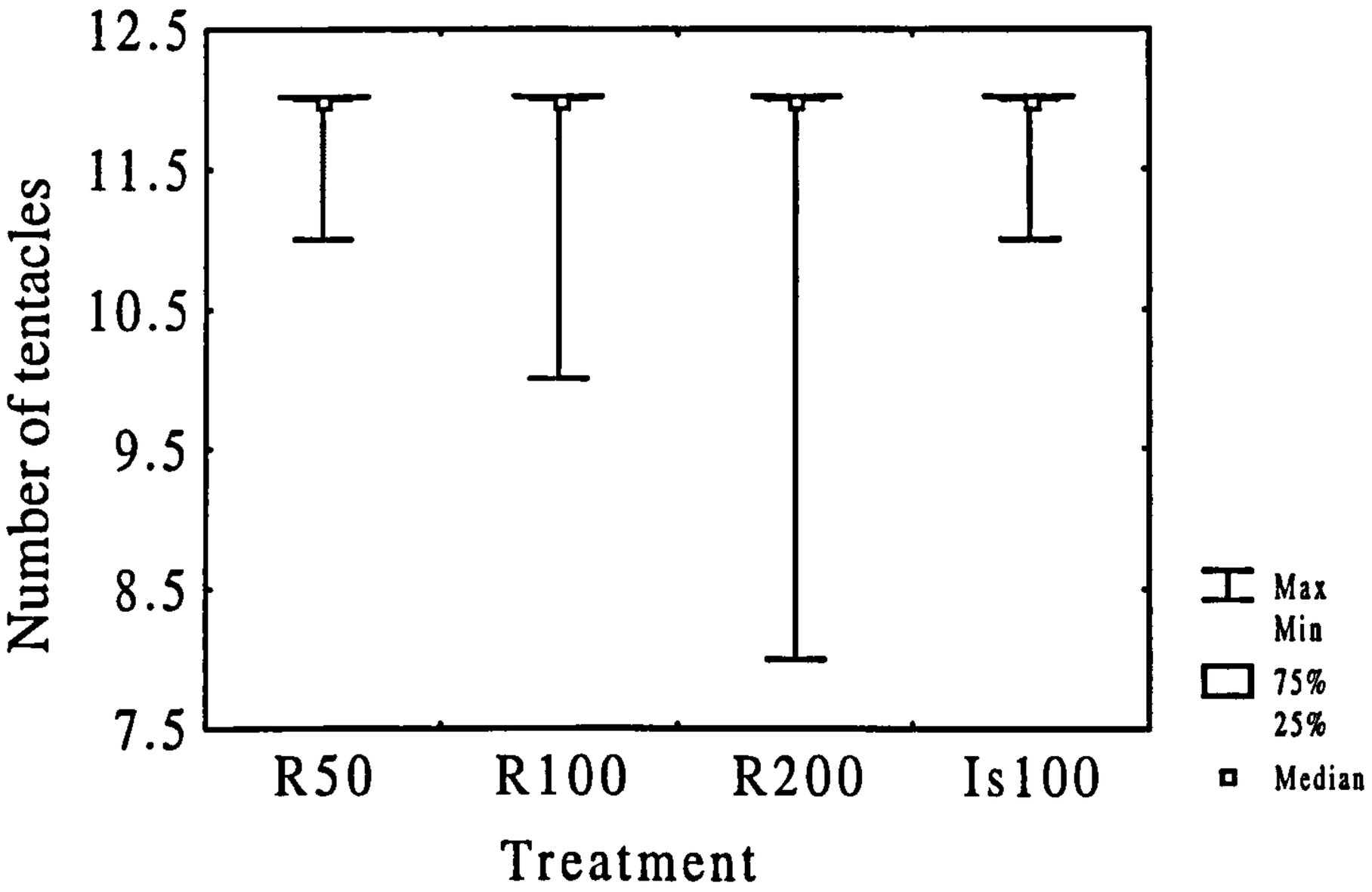


Figure 3.1. Median number of tentacles per lophophore for *C. hyalina* colonies for the 4 experimental treatments, after 56 days. *R. reticulata* treatments (R50) 50 cells μl^{-1}, (R100) 100 cells μl^{-1}, (R200) 200 cells μl^{-1} and *I. galbana* treatment (Is100) 100 cells μl^{-1}; 75% to 25% boxes define upper and lower quartiles. Lophophores (n=180), Colonies. (n=9), Genotypes (n=3).

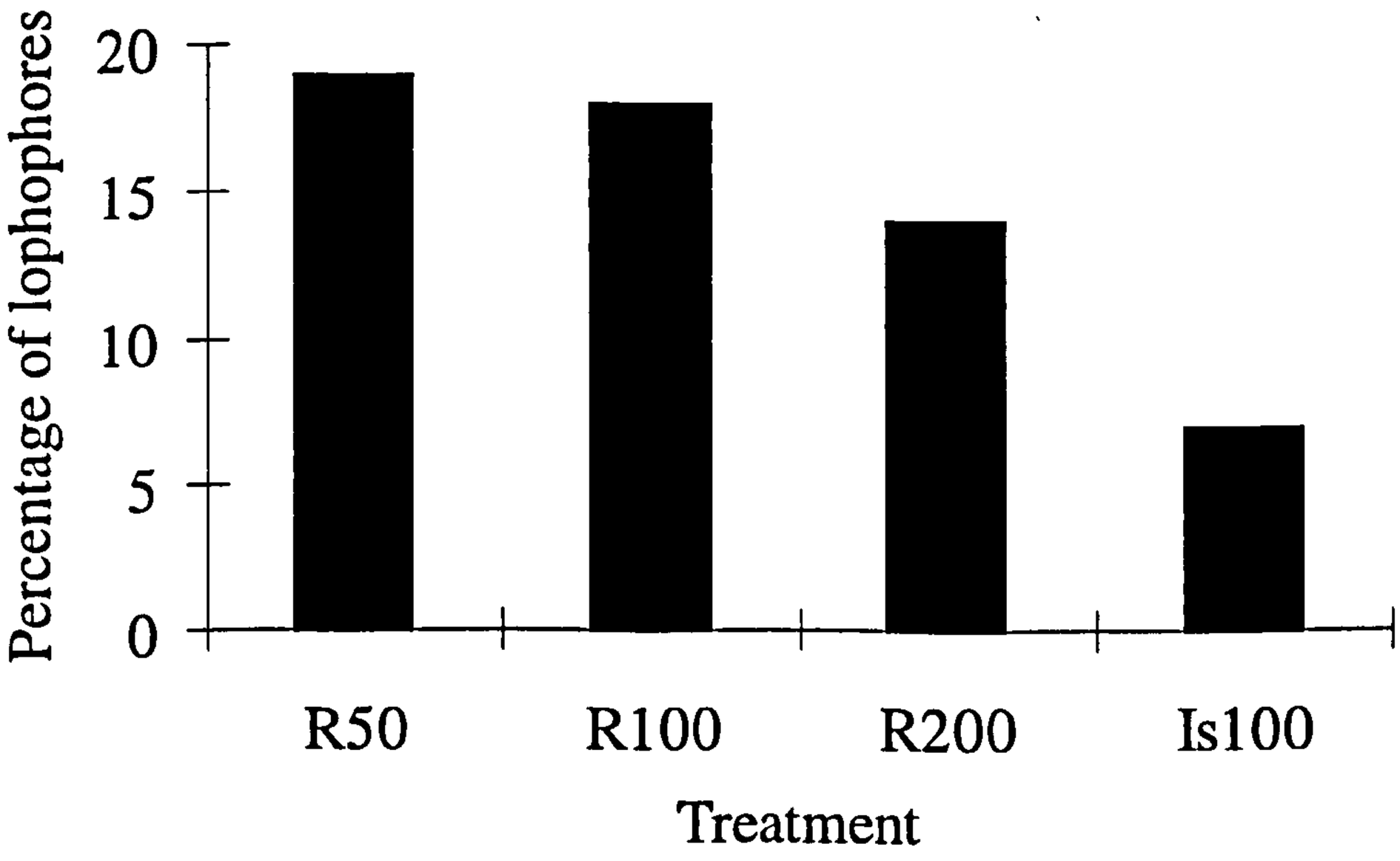


Figure 3.2. Percentage of lophophores deviating from the modal value observed for *C. hyalina* colonies for the 4 experimental treatments, after 56 days. *R. reticulata* treatments (R50) 50 cells μl^{-1}, (R100) 100 cells μl^{-1}, (R200) 200 cells μl^{-1} and *I. galbana* treatment (Is100) 100 cells μl^{-1}; Lophophores (n=180), Colonies. (n=9), Genotypes (n=3).

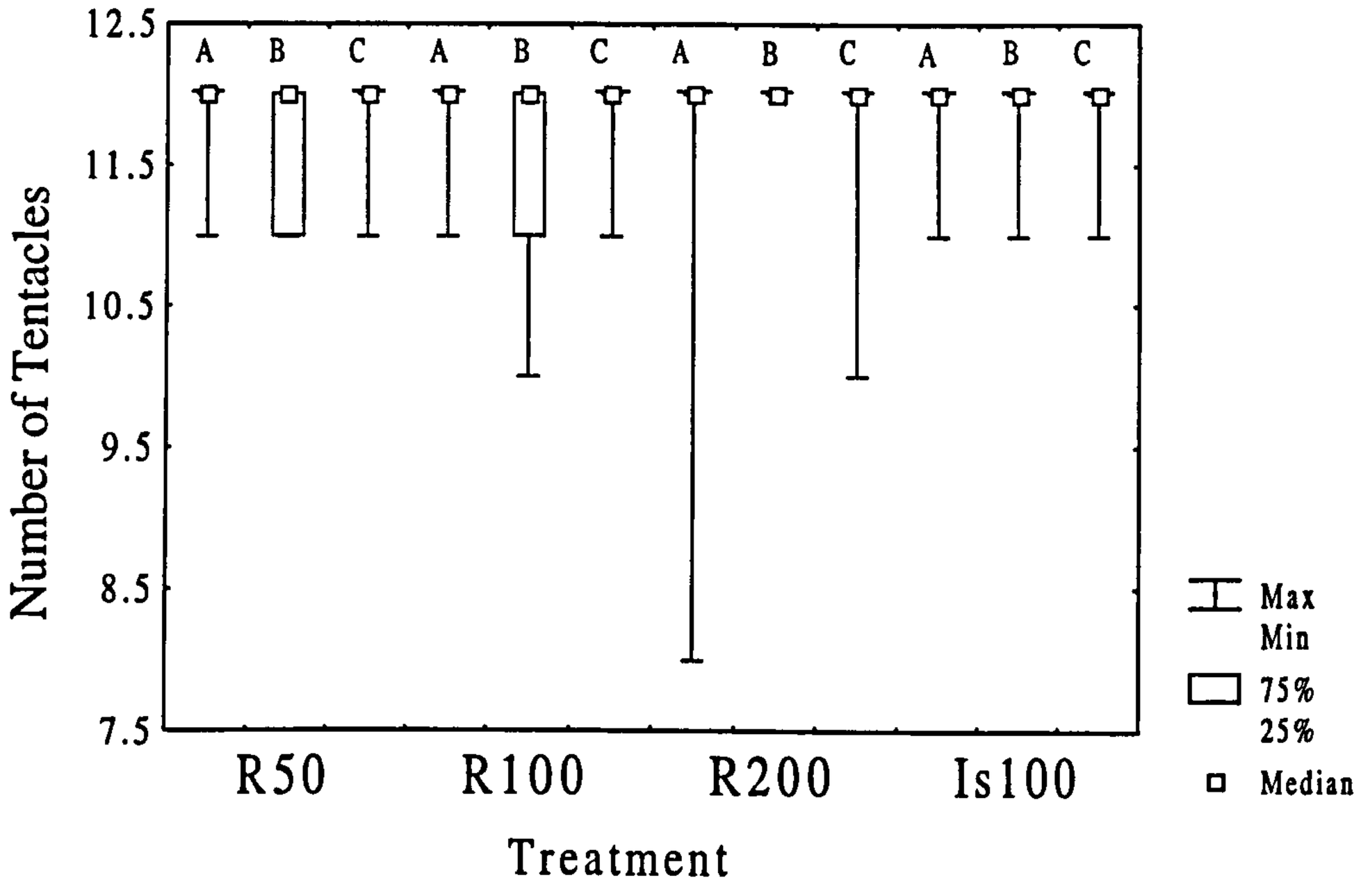


Figure 3.3. Median number of tentacles per lophophore for *C. hyalina* colonies separated for genotype and experimental treatment, after 56 days. Capital letters 'A, B and C' indicate genotype. *R. reticulata* treatments (R50) 50 cells μl^{-1}, (R100) 100 cells μl^{-1}, (R200) 200 cells μl^{-1} and *I. galbana* treatment (Is100) 100 cells μl^{-1}; 75% to 25% boxes define upper and lower quartiles. Lophophores (n=60), Colonies. (n=3).

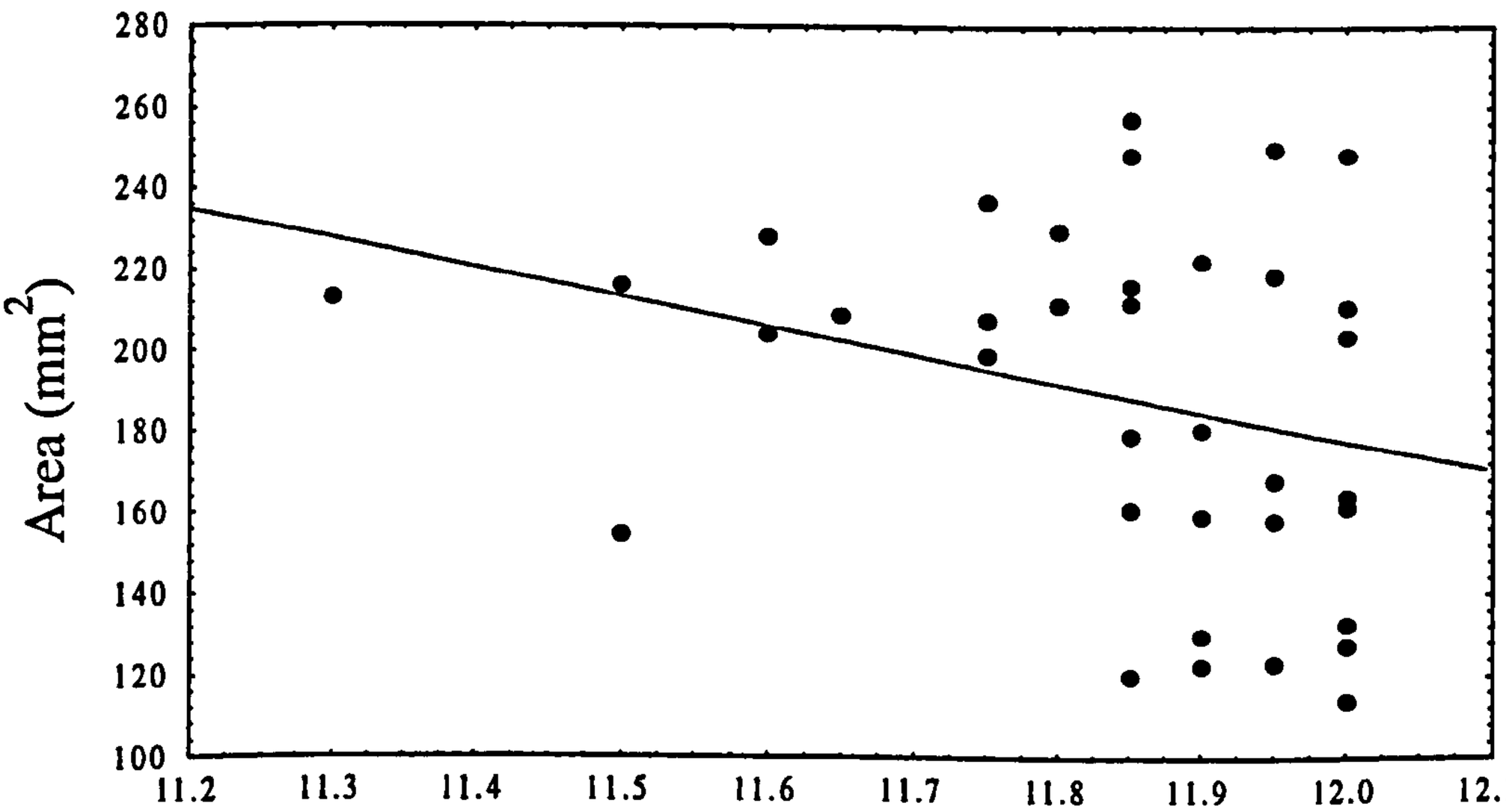


Figure 3.4. Colony area plotted against mean tentacle number (n=20) across all 4 experimental treatments after 56 days. Regression equation Y=1037.76-71.68X

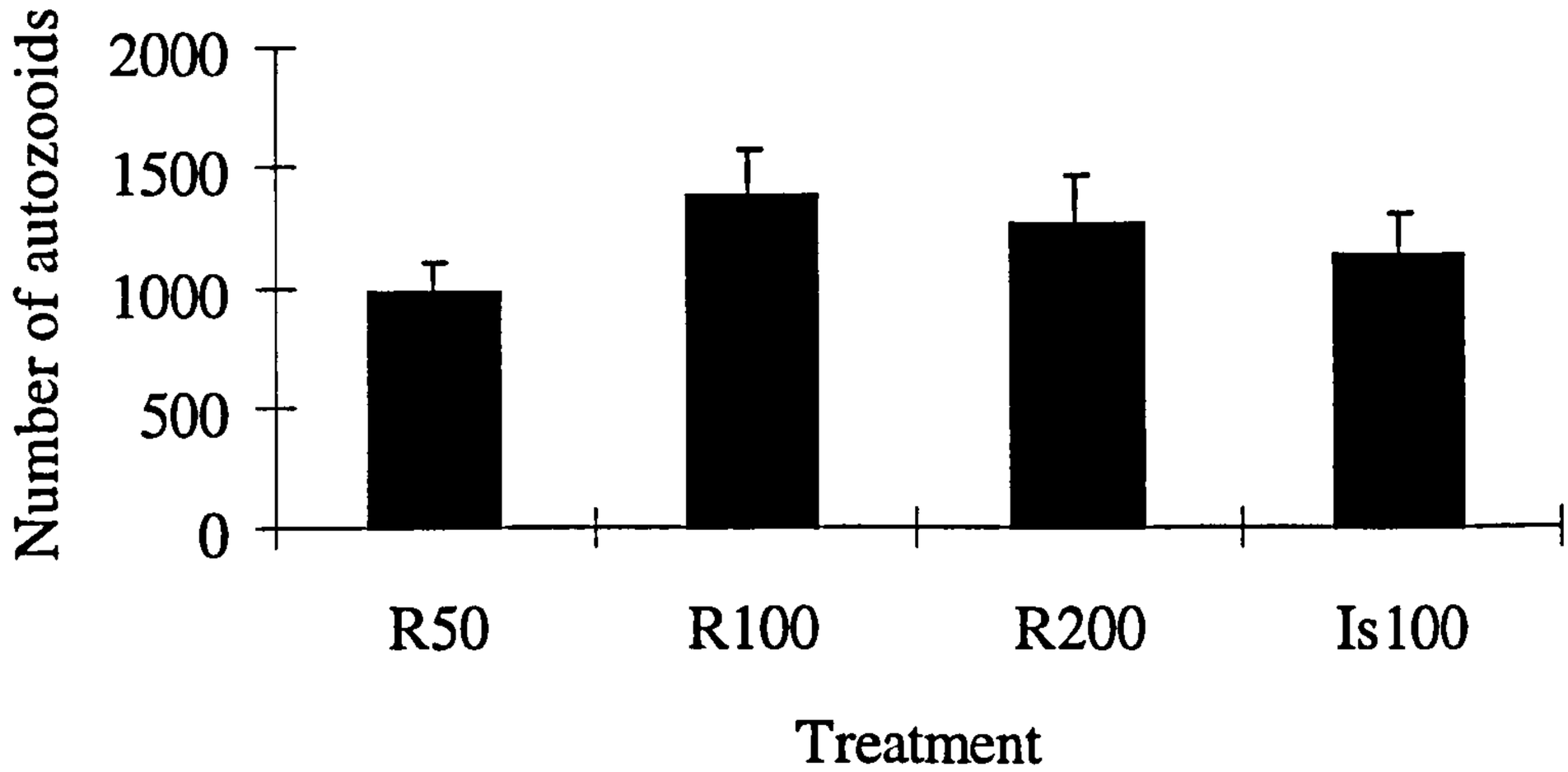


Figure 3.5. Number of autozooids (Mean + 1SD) observed for *C. hyalina* colonies in the 4 experimental treatments, after 56 days. *R. reticulata* treatments (R50) 50 cells μl^{-1}, (R100) 100 cells μl^{-1}, (R200) 200 cells μl^{-1} and *I. galbana* treatment (Is100) 100 cells μl^{-1}; Colonies. (n=9), Genotypes (n=3).

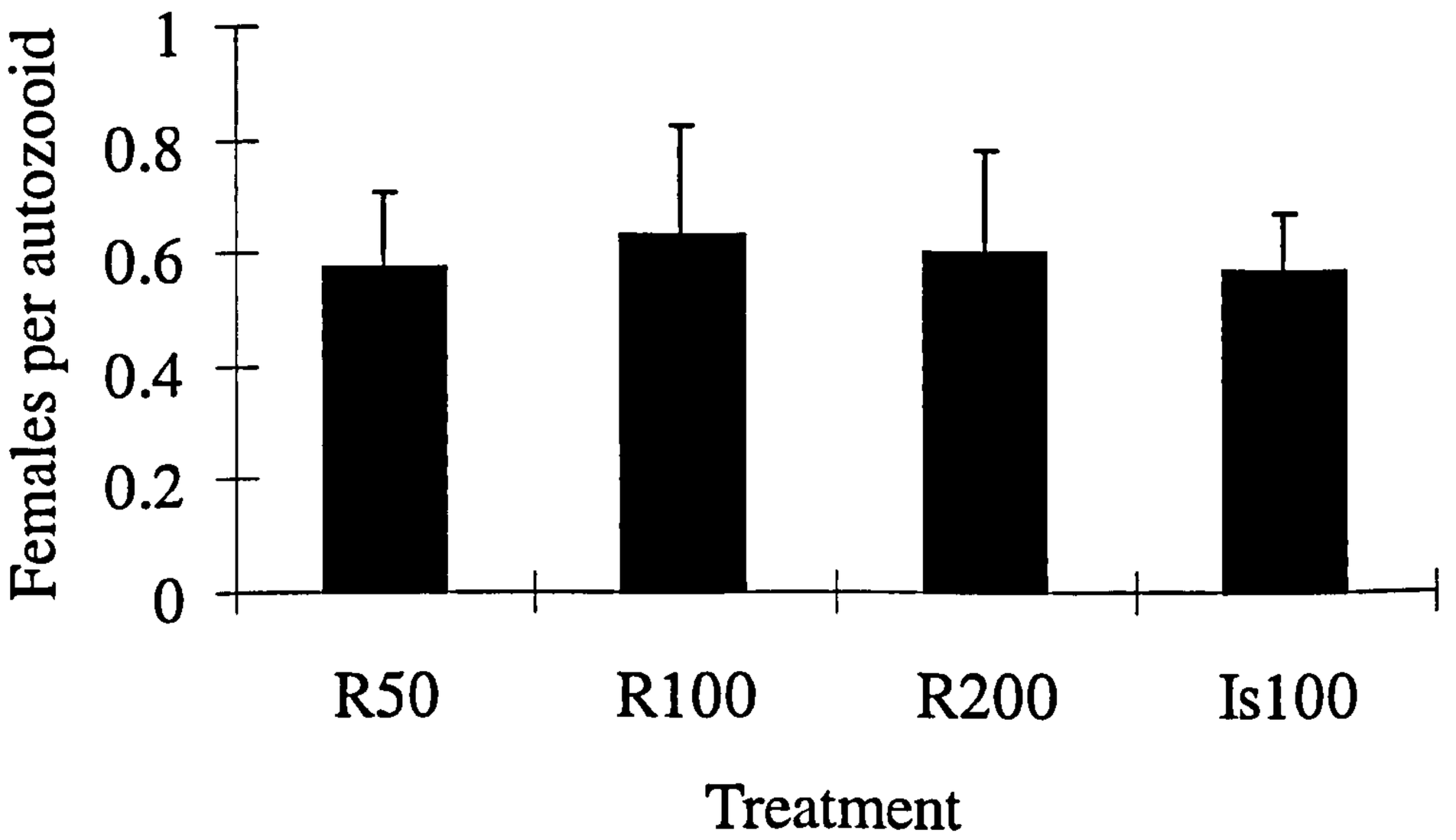


Figure 3.6. Number of females (ovicells) per autozooids (Mean + 1SD) observed for *C. hyalina* colonies in the 4 experimental treatments, after 56 days. *R. reticulata* treatments (R50) 50 cells μl^{-1}, (R100) 100 cells μl^{-1}, (R200) 200 cells μl^{-1} and *I. galbana* treatment (Is100) 100 cells μl^{-1}; Colonies. (n=9), Genotypes (n=3).

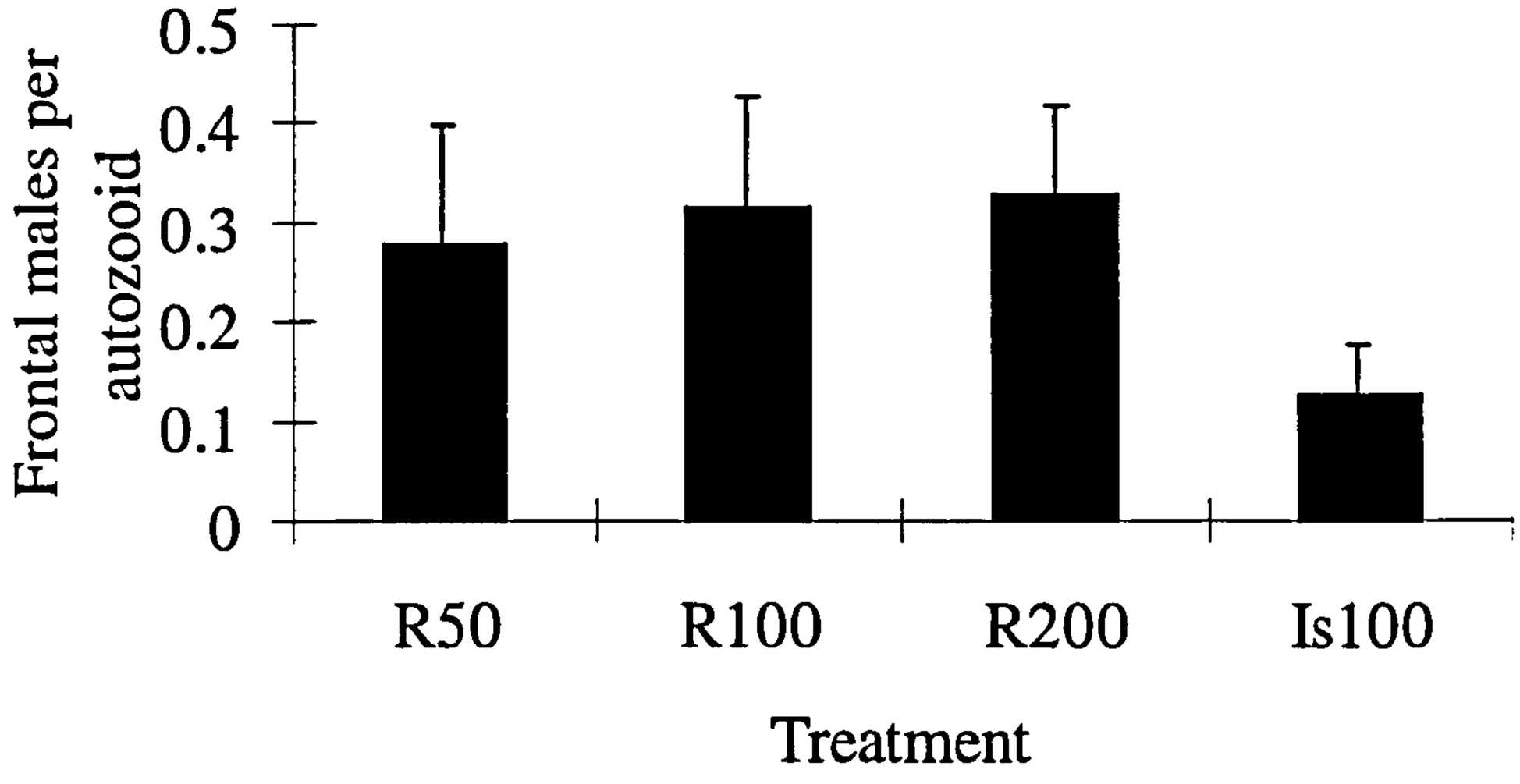


Figure 3.7. Number of frontal males per autozooids (Mean + 1SD) observed for *C. hyalina* colonies in the 4 experimental treatments, after 56 days. *R. reticulata* treatments (R50) 50 cells μl^{-1}, (R100) 100 cells μl^{-1}, (R200) 200 cells μl^{-1} and *I. galbana* treatment (Is100) 100 cells μl^{-1}; Colonies. (n=9), Genotypes (n=3).

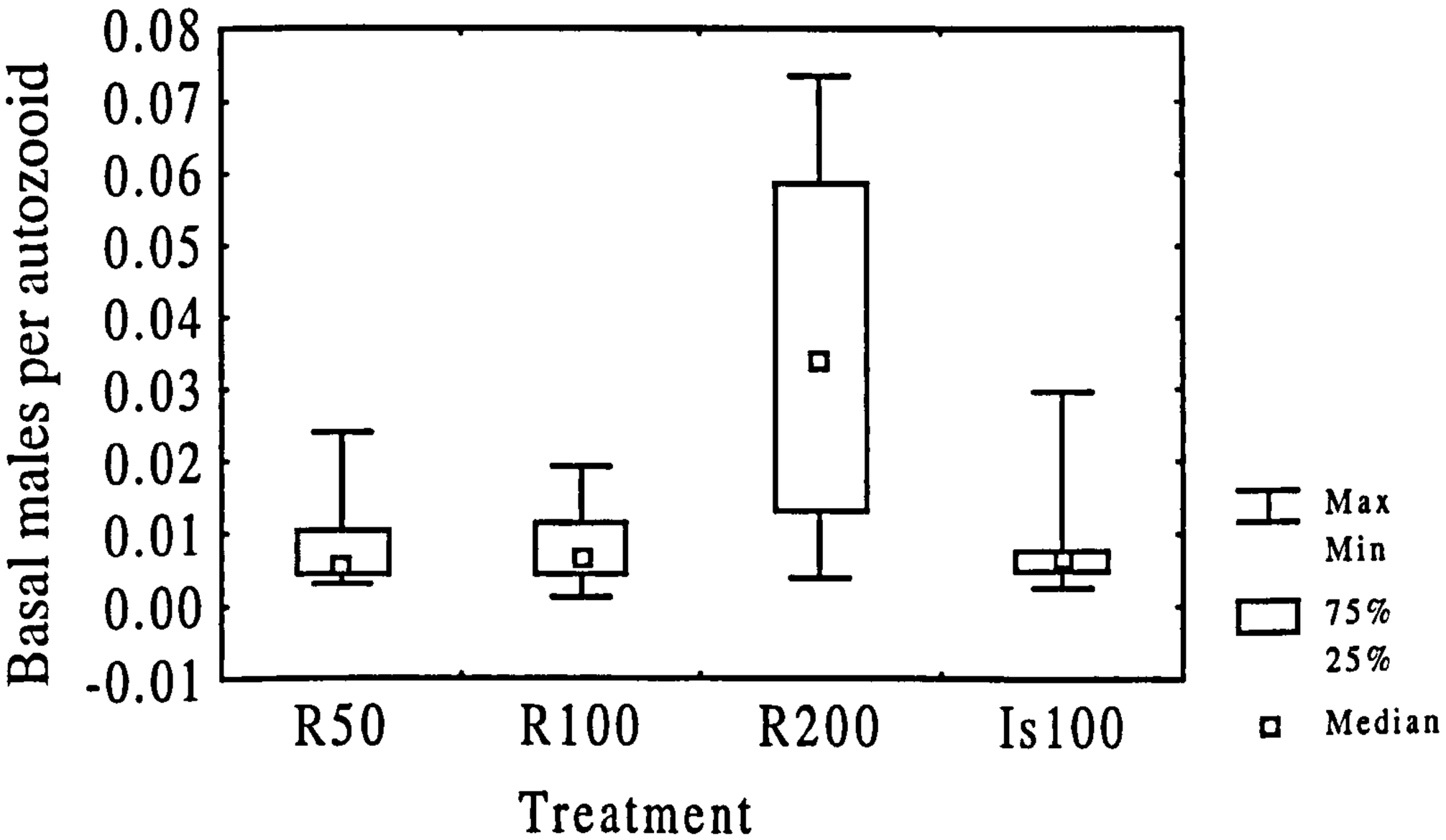


Figure 3.8. Median number of basal males per autozooids observed for *C. hyalina* colonies in in the 4 experimental treatments, after 56 days. *R. reticulata* treatments (R50) 50 cells μl^{-1}, (R100) 100 cells μl^{-1}, (R200) 200 cells μl^{-1} and *I. galbana* treatment (Is100) 100 cells μl^{-1}; Colonies. (n=9), Genotypes (n=3).

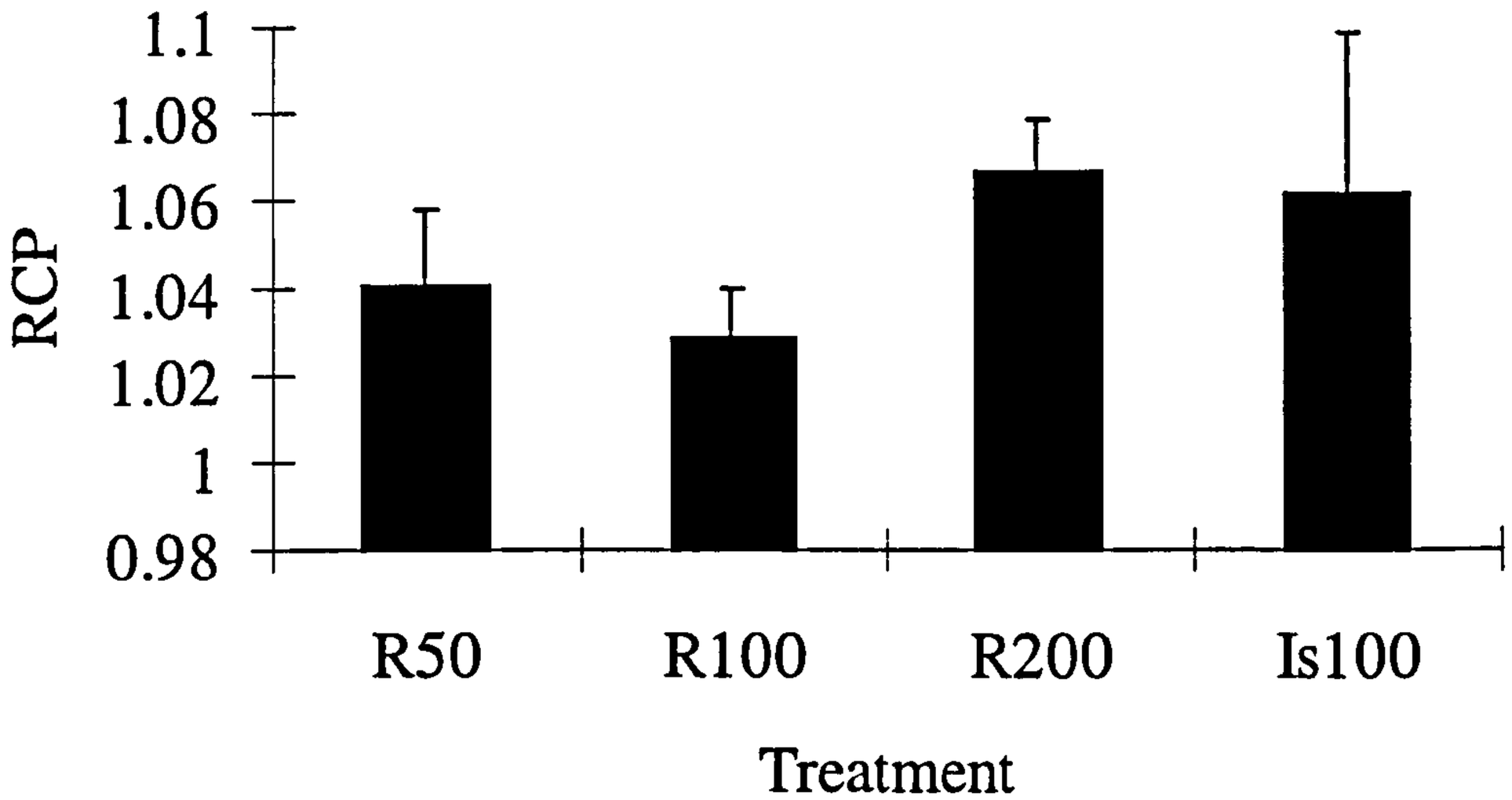


Figure 3.9. Relative colony perimeter (RCP) (Mean + 1SD) observed for *C. hyalina* colonies in the 4 experimental treatments, after 56 days. *R. reticulata* treatments (R50) 50 cells μl^{-1}, (R100) 100 cells μl^{-1}, (R200) 200 cells μl^{-1} and *I. galbana* treatment (Is100) 100 cells μl^{-1}; Colonies. (n=9), Genotypes (n=3).

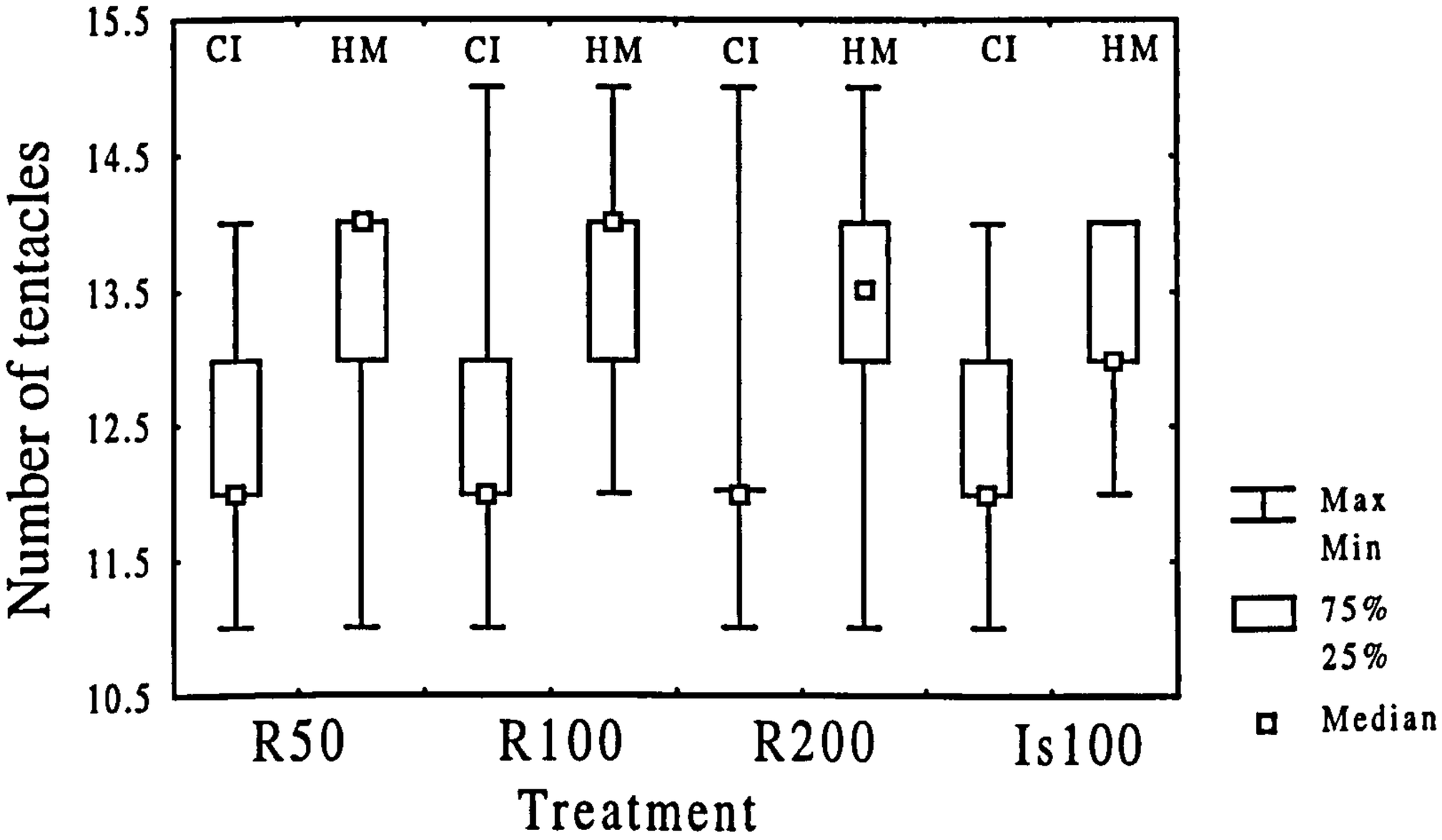


Figure 3.10. Median number of tentacles per lophophore for *E. pilosa* colonies separated on the basis of site and treatment in the 4 experimental treatments, after 56 days. CI - Church Island colonies, HM - Hell's Mouth colonies. *R. reticulata* treatments (R50) 50 cells μl^{-1}, (R100) 100 cells μl^{-1}, (R200) 200 cells μl^{-1} and *I. galbana* treatment (Is100) 100 cells μl^{-1}; 75% to 25% boxes define upper and lower quartiles. Lophophores (180), Colonies. (n=9), Genotypes (n=3).

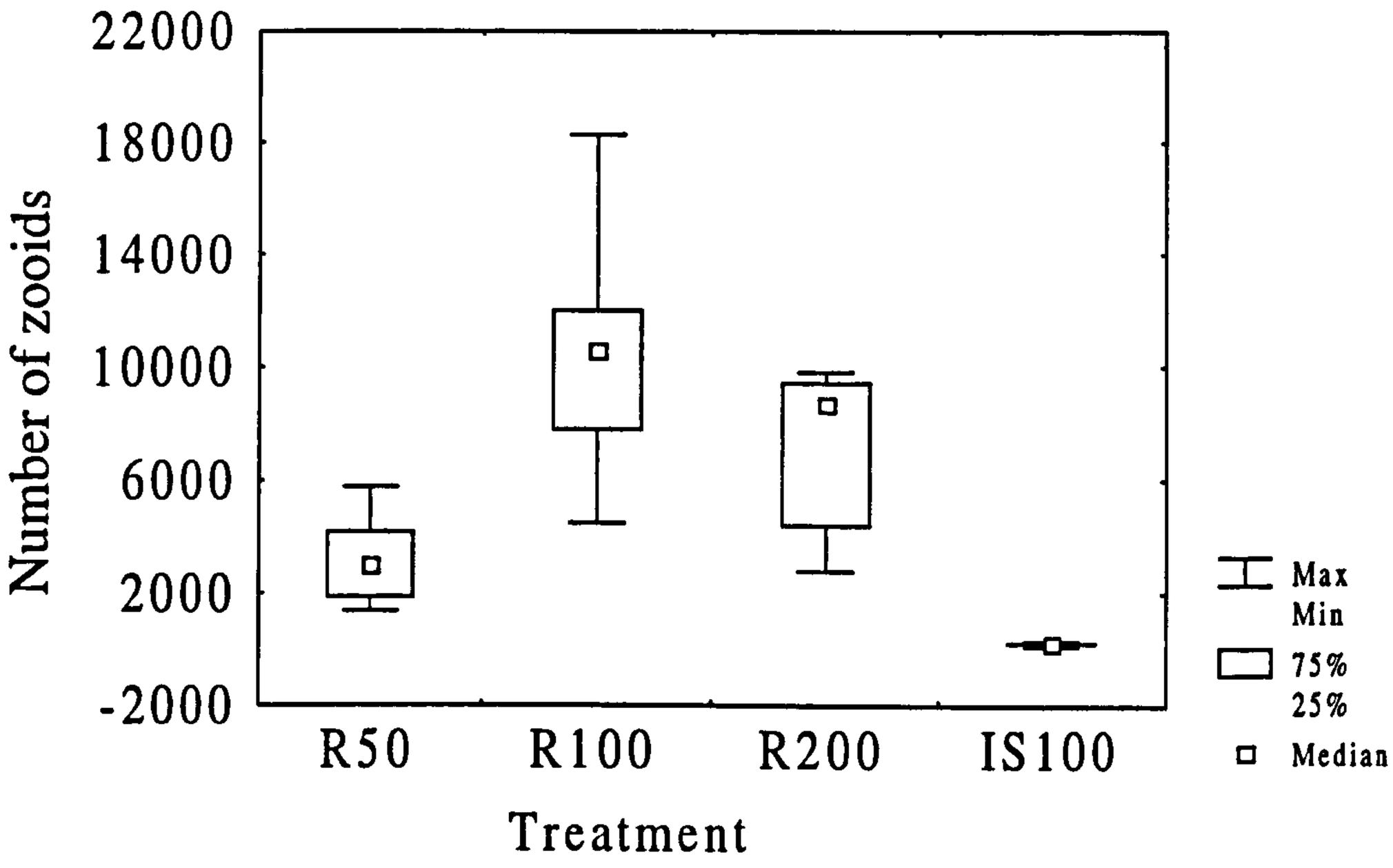


Figure 3.11. Median zooid numbers for colonies of *E. pilosa* in the 4 experimental treatments, after 56 days. *R. reticulata* treatments (R50) 50 cells μl^{-1}, (R100) 100 cells μl^{-1}, (R200) 200 cells μl^{-1} and *I. galbana* treatment (Is100) 100 cells μl^{-1}; 75% to 25% boxes define upper and lower quartiles. Colonies. (n=18), Genotypes (n=6).

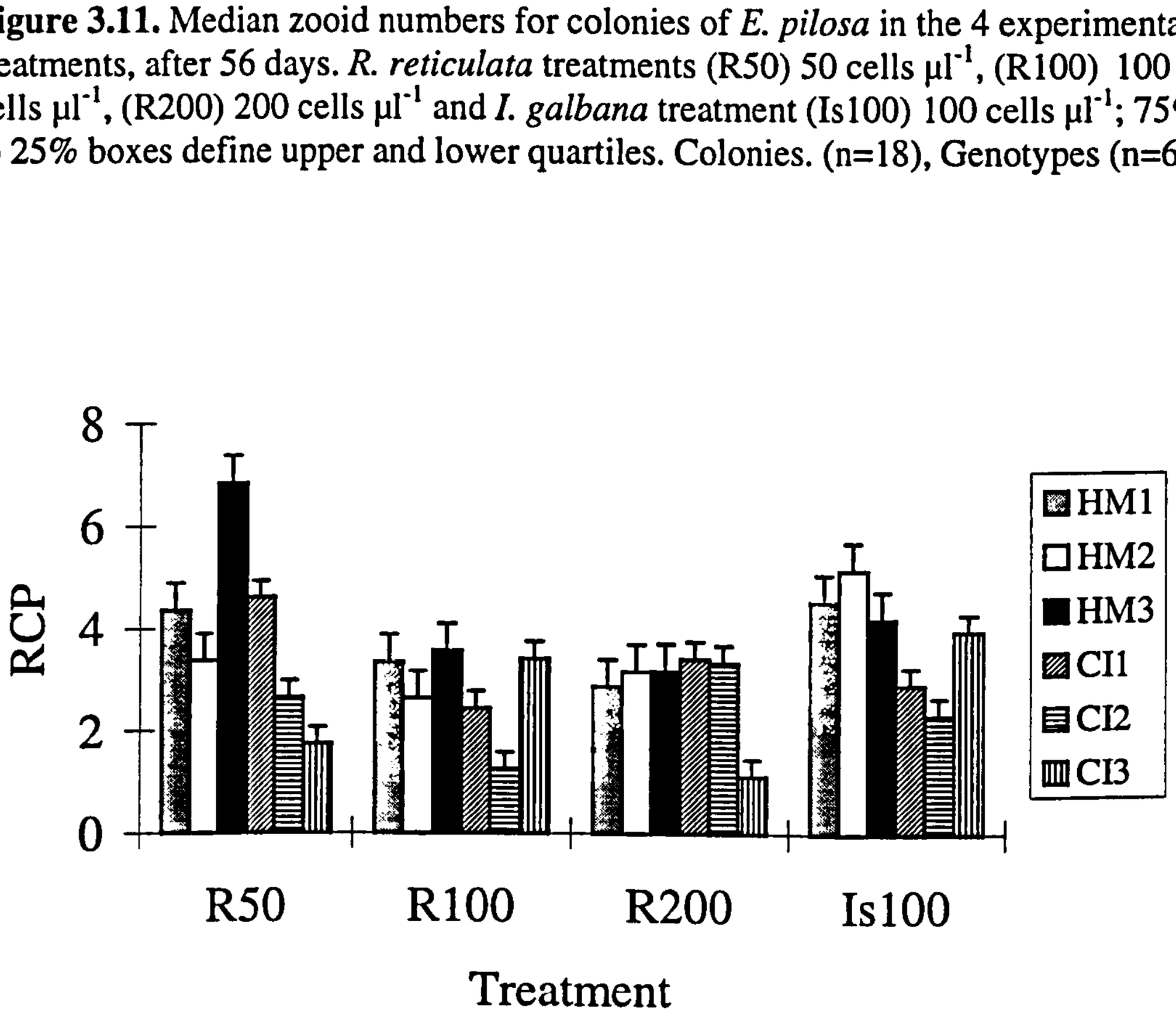


Figure 3.12. RCP values (Mean + 1SD) for colonies of *E. pilosa* separated on the basis of site and treatment in the 4 experimental treatments, after 56 days. CI - Church Island colonies, HM - Hell's Mouth colonies. *R. reticulata* treatments (R50) 50 cells μl^{-1}, (R100) 100 cells μl^{-1}, (R200) 200 cells μl^{-1} and *I. galbana* treatment (Is100) 100 cells μl^{-1}; Colonies (n=3).

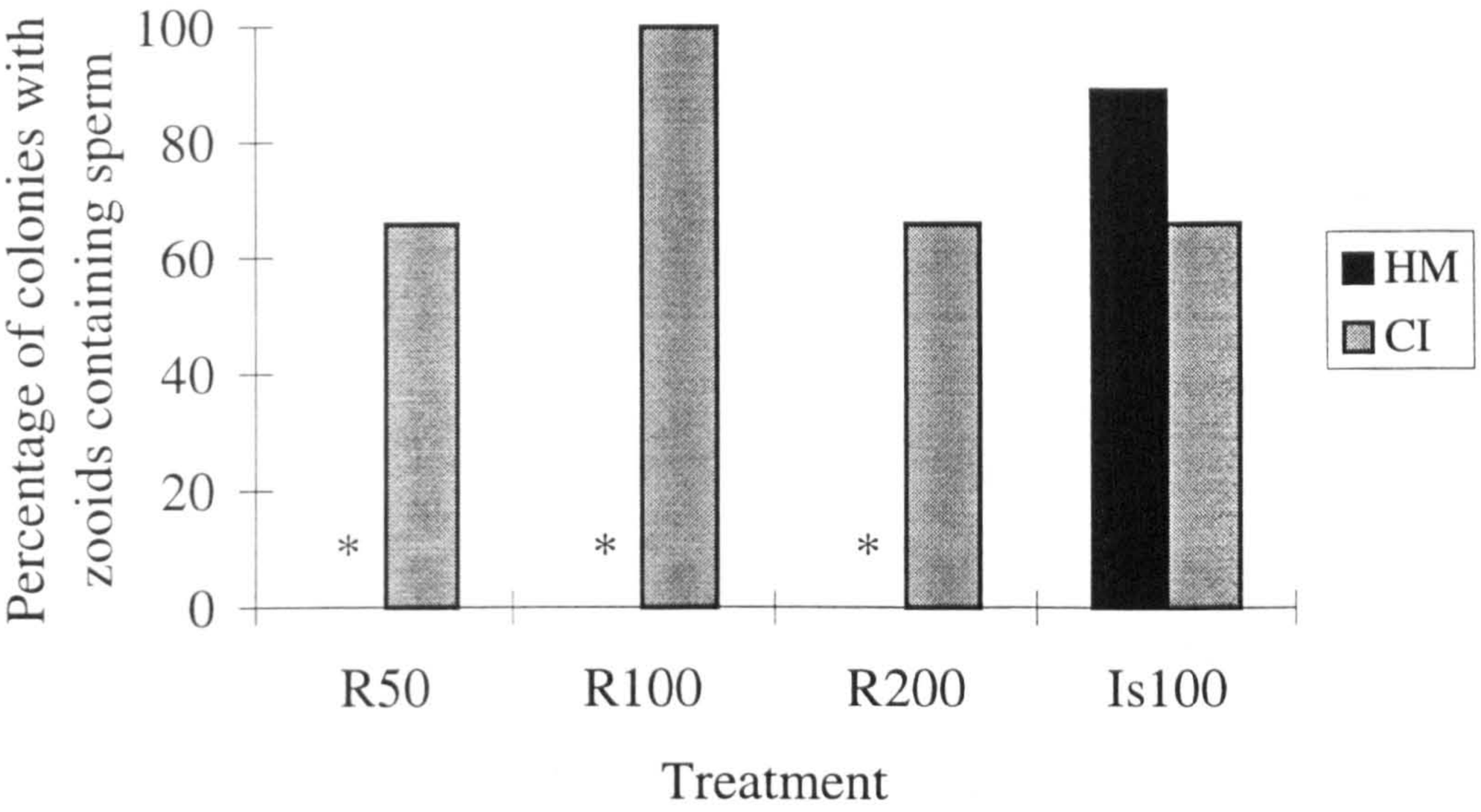


Figure 3.13. Percentage of colonies with sperm producing zooids in the 4 experimental treatments, after 56 days. *R. reticulata* treatments (R50) 50 cells μl^{-1}, (R100) 100 cells μl^{-1}, (R200) 200 cells μl^{-1} and *I. galbana* treatment (Is100) 100 cells μl^{-1}; *, no zooids observed containing spermatozoa; Colonies (n=9), Genotypes (n=3).

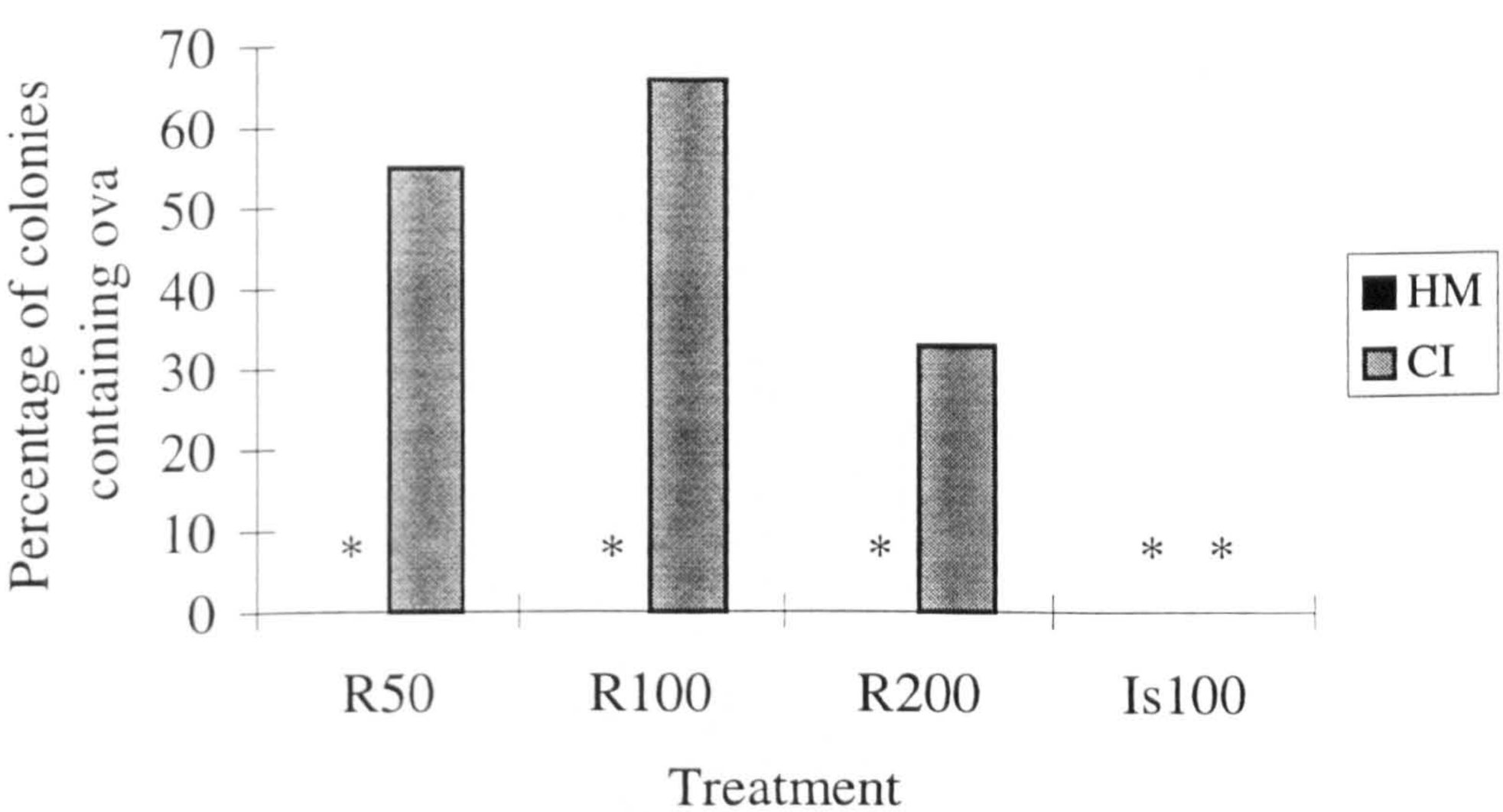


Figure 3.14. Percentage of colonies with ova producing zooids in the 4 experimental treatments, after 56 days. *R. reticulata* treatments (R50) 50 cells μl^{-1}, (R100) 100 cells μl^{-1}, (R200) 200 cells μl^{-1} and *I. galbana* treatment (Is100) 100 cells μl^{-1};*, no zooids observed containing ova; Colonies (n=9), Genotypes (n=3).

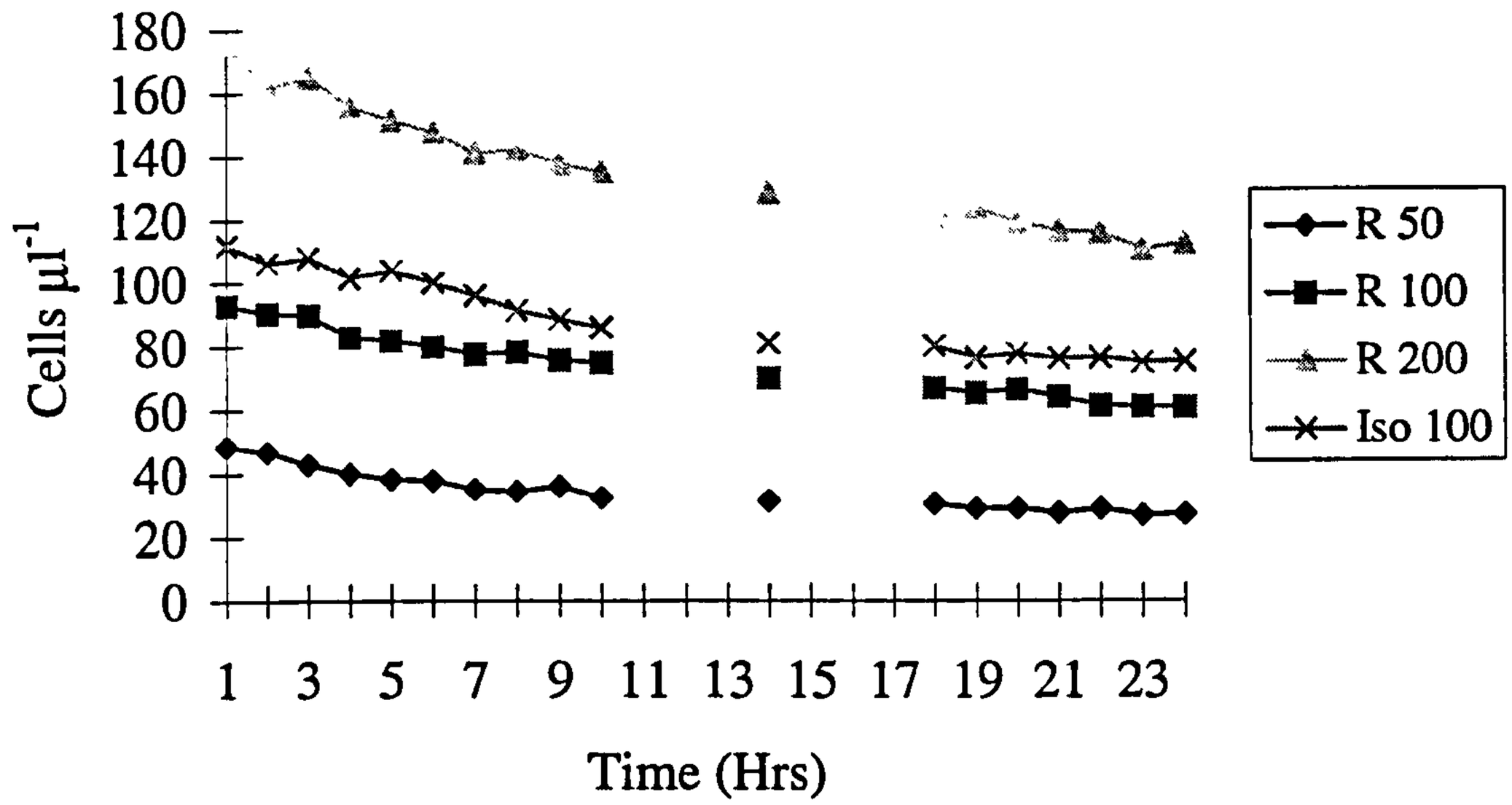


Figure 3.15. Algal concentration in the 4 experimental treatments over a 24 h period. (n=3).

3.4. DISCUSSION

C. hyalina exhibited extremely limited variation in tentacle number throughout the 4 experimental treatments. Such restricted variance appears typical of natural populations (Hayward & Ryland, 1977). The lowest variability in tentacle number was observed in the Is100 treatment. This may be due, in part, to the need of the organism to maintain high feeding current velocity in order to capture particles which are of a sub-optimal size for the filter mechanism (Riisgård & Manríques, 1997). The lowest tentacle number recorded was in the R200 treatment, possibly as a result of the stress exerted by this unnaturally high algal concentration (Riisgård & Goldson, 1998). Stressed colonies have previously been observed to produce lower tentacle numbers (Jebram, 1973).

In contrast to *C. hyalina,* the other species tested, *E. pilosa,* showed considerably more variation in tentacle number. A high degree of variation in tentacle number has been observed in natural *E. pilosa* populations by Thorpe *et al.*, (1986). In the present study, significant differences in tentacle number were observed between treatments for colonies of *E. pilosa* with generally the lowest tentacle number observed in the treatments Is100, R50 and, to a lesser extent, R200. These treatments may all be thought of as sub-optimal; R50 due to insufficient concentration to sustain maximum growth; Is100 due to decreased capture efficiency for the smaller cells of *I. galbana* or dietary deficiencies of this algal species; R200 because of the high density of cells reducing feeding efficiency (Riisgård & Manríques, 1997; Riisgård & Goldson, 1998). Jebram (1973) observed reduced tentacle number in conditions of 'malnutrition' for *Triticella korenii.* The difference in tentacle numbers observed not only between the two populations, but also between clones within these populations, indicate the obvious importance of genotype in the expression of tentacle number as a character.

No significant correlation between colony size and tentacle number was observed in either of the two species examined which contrasts with the findings for *Alcyondium* sp. (Porter *et al.*, in press). However, the methods of Porter *et al.*, (in press) differ slightly from those used in this study. The present study only counted lophophores from peripheral zooids, whereas Porter *et al.*, (in press) selected lophophores randomly from across the entire colony, which may have had a bearing

on the consequent variation in findings. Porter *et al.*, (in press) attributed the correlation of increased tentacle number with greater size to either an ageing response, older and consequently larger colonies producing more tentacles per lophophore or larger colonies requiring greater food acquisition to supply a growing colony boundary. All *C. hyalina* colonies were of the same age, being settled at the same time as larvae, and were not extremely different in size, perhaps explaining the observed lack of correlation between tentacle number and size in this species. *E. pilosa* did vary considerably in size, and probably in age due to the clones initially being cut from natural colonies of indeterminate age. Either the different ages of the genotypes used obscured any tentacle number size relationship or no advantage is conferred to larger colonies producing lophophores with higher numbers of tentacles in this species.

The importance of genotype was observed in the variation between colony form and number of females per autozooid produced in colonies of *C. hyalina*. The higher RCP values in the R200, the Is100 and, to a lesser degree, the R50 treatments indicate that colonies became less circular in form under conditions of poor nutrition due to very low cell concentration, low nutritional value of algal or excessively high cell concentration. Colonies of *C. hyalina* have been observed to become more lobate in sub-optimal environments (Hunter & Hughes, 1993b; Chapter 2). Number of autozooids, female zooids and frontal males per autozooid were highest in the R100 treatment for colonies of *C. hyalina*, demonstrating the importance of algal concentration and algal species on growth parameters. Basal male production was highest in the R200 treatment, this probably being due to the formation of these zooid types in response to stressful conditions. Previously, increased production of basal males has been associated with extremely poor nutrition (Hunter & Hughes, 1993b). However, toxicant stress also appears to result in large numbers of basal males being formed (Chapter 2). It must be surmised that the R200 treatment, through the excess of algal cells interfering with lophophore feeding behaviour, heavily stressed the *C. hyalina* colonies. The switch in production of autozooids to basal males in stressful environments may well increase the likelihood of the perpetuation of parental genes, by producing large numbers of relatively physiologically inexpensive gametes prior to the demise of the colony.

Colonies of *E. pilosa* also showed significant variation between genotypes for RCP. The colonies with the least regular form occurred in the R50 and Is100 treatments. A multiserial stellate colony form has been attributed to rapid growth of *E. pilosa* colonies (Okamura, 1992; Bayer & Todd, 1996). However, in nutritionally deficient conditions bryozoan colonies have been found to produce uniserial runners (Winston, 1976; Jebram & Rummert, 1978). The colonies which exhibited the highest RCP values appeared to have just begun to form such runners. It seems likely that the algal concentrations in the R50 and Is100 treatments were initially sufficient to promote colony growth. However, with the observed increases in colony size, over the course of the experiment, the nutritional requirements of the bryozoans were no longer being met. Consequently, at the end of the experiment runners were beginning to be formed.

The influence of site for *E. pilosa* colonies was very pronounced with respect to sexual allocation. CI colonies produced zooids containing sperm across the 4 treatments, some variation amongst genotypes within this group was also detected. The highest production of spermatozoa was observed in the R100 treatment, this probably being due in part to larger size of the colonies and lower stress related to cell concentration. In stark contrast to the CI group, the HM group produced gametes in only the Is100 treatment. This may be a result of this treatment being particularly stressful initiating sperm production or *I. galbana* providing a nutrient essential to the formation of gametes in this group, which is lacking in *R. reticulata*. Bayer *et al.*, (1994) expressed concerns over the effectiveness of a mono-diet for *E. pilosa* producing optimal growth and reproduction. Curiously, it appears as if *I. galbana* provides an essential component necessary for gamete formation in colonies from the HM population, and has proved to be effective in conjunction with *R. reticulata* (Chapter 4). Results from this study indicate that, although *I. galbana* seems to provide an essential dietary component for gamete formation in some genotypes, as a mono diet it produces a very reduced colony growth. No ova were produced in the Is100 treatment by either HM or CI colonies, however, this is probably due to their small size, less than 315 zooids. Hughes (1986) found that in natural populations ova were not produced in colonies under 850 zooids in size. Cancino *et al.*, (1991) found a diet of *I. galbana* and *Dunaliella tertiolecta* was sufficient for colonies of *Membranipora isabelleana* to produce mature oocytes.

In conclusion, tentacle number appears to lack any significant plasticity in *C. hyalina* in relation to diet. *E. pilosa* exhibits considerable variation in tentacle number, the majority of which can be assigned to genotypic variation. Nevertheless, dietary quality does appear to influence tentacle number such that poor nutrition results in reduced tentacle number. However, extreme caution must be exercised in interpreting these results in terms of algal concentration. The single dose of algae used to obtain a concentration over a 24 h period is far from optimal in examining the influence of different feeding regimes. A large proportion of algal cells fall out of suspension and the remaining quantity is subject to removal by the animals themselves, particularly in the latter part of the experimental period when colonies have grown considerably. Over 24 h period concentrations probably range from the intended treatment to around zero. A wide range of laboratory based studies to examine plasticity in tentacle number in terms of local competition, feeding period and flow environment may prove considerably more fruitful than the present study. The effects of diet on other growth parameters in both species has been highlighted as well as the importance of genotype. Finally, the addition of *I. galbana* is recommended to supplement *R. reticulata* based diets for *E. pilosa*.

CHAPTER 4.

WAVE INDUCED MORPHOLOGICAL PLASTICITY IN *ELECTRA PILOSA*

4.1. INTRODUCTION

Phenotypic plasticity can be defined as the capacity of an organism to alter phenotypic expression of a genotype in response to an environmental variable, subsequently resulting in increased fitness. Natural selection is generally considered to drive the evolution of phenotypic plasticity (Schlichting, 1986; Stearns, 1989). The underlying genetic mechanism for this phenomenon has, however, caused considerable debate (Scheiner,1993a,1993b; Schlichting & Pigliucci, 1993; Via, 1993). More recently, it has been concluded that phenotypic plasticity is a trait which is in itself an adaptation (Via *et al.*, 1995). Phenotypically plastic traits can be found in numerous taxa, affecting morphology and life histories in many ways (Schlichting, 1986; Stearns & Koella, 1986; Stearns, 1989; Houston & McNamara, 1992; Newman, 1989; Gotthard & Nylin, 1995; Merilä, 1997).

Bryozoa provide some excellent examples of life history and morphological modifications resulting from biotic and abiotic stimuli. Instances of life history variation, such as change of reproductive timing, occur in *Membranipora membranacea* in reaction to contact with other colonies (Harvell & Helling, 1993). In *Celleporella hyalina* variations in sexual allocation can be induced by temperature, dietary or toxicant stress (Hunter & Hughes,1995; Chapter 2). Examples of morphological modification include variation in zooid size and the production of defensive spines. The former trait has been observed in the bryozoans *Conopeum reticulum* and *Electra pilosa* in response to temperature change (Menon, 1972; Okamura, 1987), while the latter occurs in *M. membranacea* as a response to grazing by nudibranchs and in *E. pilosa* when threatened by overgrowth (Stebbing, 1973a; Yoshioka, 1982b; Harvell, 1984). More recently, wave related abrasion of *E. pilosa* colonies has been found to cause an increase in length of median proximal spine formation (Bayer *et al.*, 1997).

The discrete production of extended spines in peripheral zooids of *E. pilosa*

colonies under threat of overgrowth was observed by Stebbing (1973a). Colonies with a sparse covering of extended spines over the whole surface were also found, but no satisfactory explanation was offered for this. The occurrence of some short spined (≈25 μm) colonies, with others possessing spines more than 80 times this length, giving a 'hairy' appearance, lead to the creation of a taxonomic subspecies by Hincks (1880).

Copious spine production has been observed on *E. pilosa* colonies collected from fronds of *Fucus serratus* from sites of high wave exposure, without any obvious signs of imminent threat of overgrowth (AJG. pers. obs.). Recent experiments have shown that nudibranchs do not induce spine formation in this species (M. Bayer pers. comm.). This presents the possibility that the formation of 'hairy' colonies is induced through exposure to conditions associated with a heavy wave action. It seems likely that zooids are exhibiting a polyphenic form of phenotypic plasticity, in that a gradual environmental change results in an abrupt switch between two discrete phenotypes (i.e. zooids with 'long' or 'short' proximal spines). Polyphenic phenotypic plasticity has also been referred to as 'developmental conversion' by Smith-Gill (1983).

Wave action exerts a suite of stresses on filter feeding epiphytic organisms which, in turn, could act as triggers for the production of polymorphisms, such as changes in spine length. For example, Whitehead *et al.* (1996) induced increased spine number in the ctenostome *Flustrellidra hispida*, by exposing colonies to turbulent flow conditions, while also observing a genetic component to spinosity. *F. hispida* larvae grown under identical laboratory conditions were found to exhibit the same degree of spinosity as their maternal colony. However, clone mates exposed to different degrees of turbulence, showed a significant increase in spine number in more turbulent treatments (Whitehead *et al.*, 1996). Reverter Gil (1994) had previously observed that increased wave exposure in natural populations resulted in more spinous colonies of *F. hispida*.

There are likely to be substantial energetic costs associated with 'long' spine production in *E. pilosa*, as well as, increasing drag on colonies in highly dynamic environments (Denny *et al.*, 1985). Extended median proximal spines must therefore offer considerable benefit to the organism in order compensate for the adverse effects. The aims of this study are to identify the cue necessary to initiate extended

spine growth, and to propose possible functional advantages of 'long' spines.

In order to accomplish these goals, natural populations of *E. pilosa* were examined to assess the extent of naturally occurring spine variation in two areas of contrasting wave exposure. Reciprocal transplants were then undertaken between the two sites, in order to establish the importance of natal location in the polymorphic response. Laboratory investigations were then targeted at recreating the trigger for the dramatic increase in spine length in some zooids and to assess the importance of genotype in the expression of this character.

The possible wave related cues of turbulence and abrasion, both particulate and from local surfaces, were examined as potential triggers for zooid polymorphism. Water turbulence as a cue for spine production has previously been mentioned (Whitehead *et al.*, 1996). The effects of abrasion on epiphytic colonies are likely to be of major importance particularly with regard to relatively fragile filtering apparatus such as the lophophore used by bryozoans. The presence of suspended particulate matter is thought to initiate lophophore retraction, in an effort to protect the tentacles against mechanical damage (Best & Thorpe, 1996). Lophophore retraction results in reduced feeding time which is particularly important considering the energetically marginal existence that suspension feeding is thought to offer (Jørgensen, 1966, 1975). Retraction of the lophophore is also induced by direct contact with neighboring substrata. Mechanical forces of less than 1 mg applied to *Membranipora membranacea* lophophores cause retraction of that individual lophophore. If mechanical forces of approximately 10 mg are applied to the frontal membrane of an individual zooid, the surrounding zooids also retract their lophophores (Thorpe *et al.*, 1975a, 1975b; cited in Best & Thorpe, 1996). For epiphytic colonies the most likely source of substrata abrasion is from neighbouring algal fronds. Intertidal algae, such as *F. serratus*, experience a great deal of contact within and between plants, due to wave related oscillations (Denny, 1988; Vogel, 1994).The production of spines extending well above the height of the feeding lophophores, may provide protection against direct physical contact with larger particulates or local substrata, enabling feeding to continue unhindered. Recent findings suggest, that under naturally occurring temperate region phytoplankton concentrations, bryozoan colonies feed continuously, further emphasizing the need to protect and maintain an everted lophophore (Riisgård & Goldson, 1997).

Finally, particle paths over colonies, possessing either 'long' or 'short' spines were observed to appraise the effect of spine length on velocity of potential food items and the consequent lophophore capture efficiency at increased flow speeds. Particle capture efficiency has been shown to decrease with increasing flow velocity in encrusting bryozoan species (Okamura, 1985, 1988, 1992). Flume observations of *F. hispida* colonies indicate that at high velocities everted lophophores may be bent over by the current, probably preventing efficient feeding (Whitehead *et al.*, 1996).

Surface spines are thought to increase the boundary layer thickness over bryozoan colonies (Riedl & Forstner, 1968). In high flow velocities the increase in the boundary layer thickness, may enable lophophores to be extended or slow food particles sufficiently to allow their capture by colony feeding currents.

4.2. MATERIALS AND METHODS

4.2.1. Natural spine length variation

4.2.1.1. Comparison of colonies from two study sites

Colonies of *E. pilosa* were randomly sampled from two sites of contrasting exposure on the 10th September 1995, Church Island (CI), Menai Strait, North Wales (53° 13' N 4° 11' W) and Hell's Mouth (HM), Abersoch, North Wales (52° 17' N 4° 33' W) (Fig. 4.1). CI has very limited wave exposure but strong tidal currents of speeds up to 1.2 m sec^{-1} (Harvey, 1968; Wood & Seed, 1992) while HM experiences waves of over 2.5 m for 10% of the winter months (Evans, 1995). Colonies of *E. pilosa*, collected on *F. serratus* were brought back to the laboratory for examination. The length of the median proximal spine was measured for 10 zooids from 20 colonies for each site. A Wild high power dissecting microscope with eye-piece graticule was used to obtain proximal spine length measurements. Spine measurement was only undertaken if the selected zooid was at least 10 zooids distant from the colony periphery, to avoid recording spines that were not fully developed. To examine the progression of spine growth with zooid age, spine lengths were also recorded for 20 zooids, in sequence, working inwards from the colony periphery.

4.2.1.2. Degree of exposure

To obtain a qualitative measure of the degree of water movement between the two study sites (CIA and HM), calcium sulphate (plaster of Paris) spheres (5 cm dia.) were tied to fronds of *F. serratus*. Three spheres were placed at each site, in areas where colonies exhibited spine lengths typical for that area (Fig. 4.1). A survey of the islets associated with CI revealed one small area between Ynys Welltog and Ynys Benlas where colonies of *E. pilosa* exhibited unusually long spines (Fig. 4.2 & 4.3). Three plaster spheres were also placed in this area CI(Z). Spheres were subjected to 24 hours exposure, being placed out at low spring tide on 16th April 1996 and collected at the same time the following day. Manufacture of the plaster of Paris spheres was according to Muus (1968). However, because of the difficulties associated with laboratory calibration of the spheres in relation to specific flow velocities (Thompson & Glenn, 1994), the measurements obtained from weight loss of the spheres after

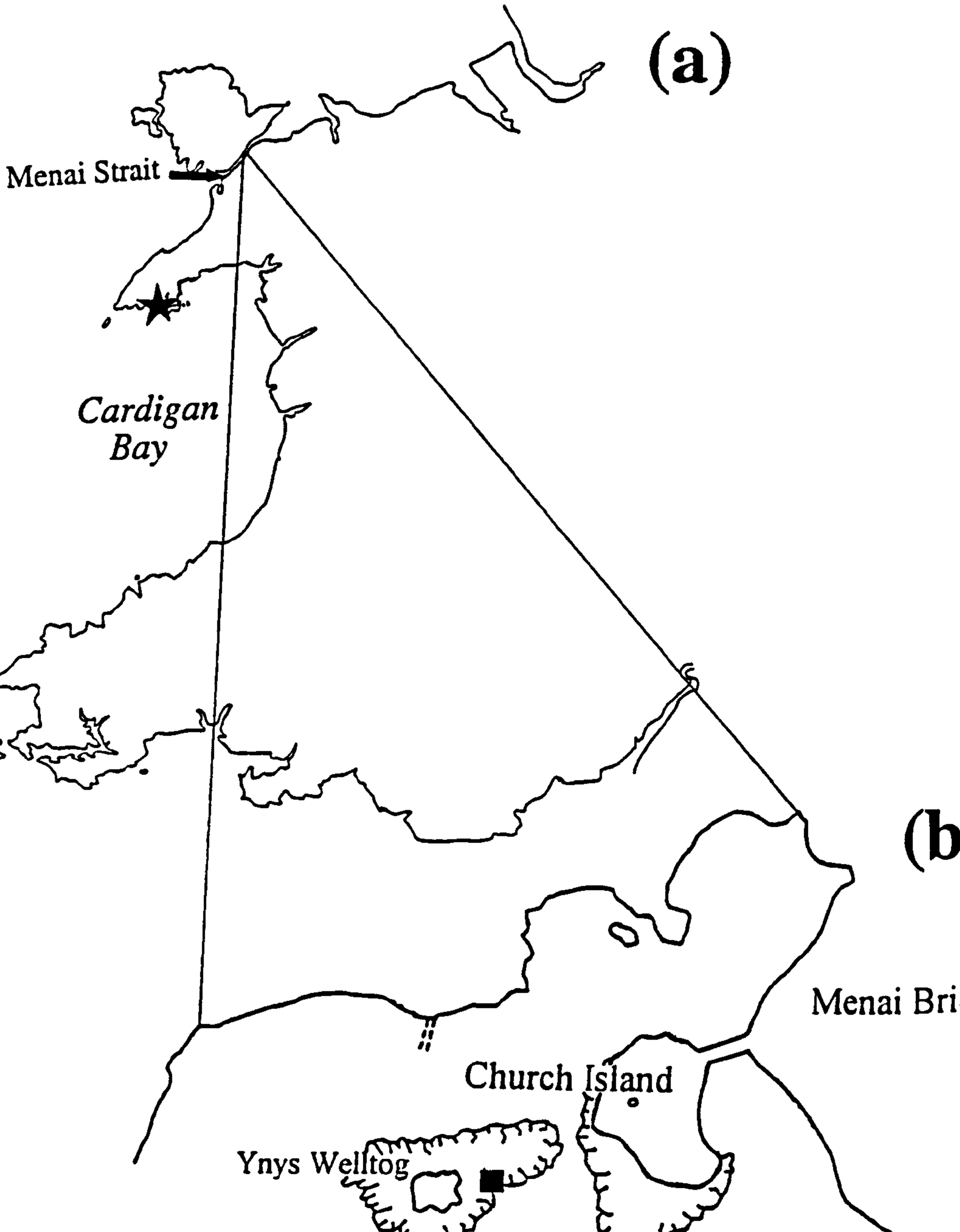


Figure 4.1. Site map of sampling areas, (a) Wales, (b) Church Island, Menai Strait, ★ = Hell's Mouth (HM), ■ = Church Island (CI A), ● = Church Island (CI Z).

Figure 4.2. Church Island, Menai Strait location of site CIZ and CIA indicated.

Figure 4.3. Site CIZ, showing gully and high degree of water movement.

rinsing and drying, were only used in a qualitative comparison of exposures between the sites.

4.2.2. Turbulence

Methods for the propagation of experimental colonies of *E. pilosa* are given in Bayer *et al.*, (1994). HM and CI colonies were used to generate a number of clones. Ramets were established on separate glass microscope slides (7.5 cm x 3.9 cm). Each individual was reduced to 20 healthy zooids, using a fine mounted needle (0.30 mm x 30 mm). Plastic slide racks (92 mm x 80 mm x 21 mm) holding 6 slides, supporting 3 genets from each site, were suspended in a 3 litre Polyethylene Cola bottle with the neck section removed. A 60 ml syringe with the plunger removed, was mounted centrally, using super glue at the base of the container, inside which was an airstone supplied by 0.5 cm dia. tubing (Fig. 4.4). Vessels contained 2.5 litres of 0.2 µm, UV-irradiated seawater to which was added *Rhinomonas reticulata* (75 %) and *Isochrysis galbana* Parke (25%) algal diet to achieve a final concentration of 100 cells μl^{-1}. Slots in the base of the syringe barrel allowed water to be drawn into the syringe, as air exited through an enlarged hole at the top of the syringe, thus creating a turbulent flow. The plastic rack holding the colonies was positioned directly above the current. Two turbulent flow environments were established, low turbulence (50 cm^3 air min^{-1}) and high turbulence (1500 cm^3 air min^{-1}). Colonies were randomized within treatments daily, to eliminate container and positioning effects. Colonies were cleaned with a fine nylon artists' brush at weekly intervals to remove fouling. Containers were emptied and rinsed out daily and fresh seawater and algal suspensions were added. Salinity (35 ‰) and pH (7.90) remained constant between treatments and over time. Containers were maintained at 18 (±1)°C in a constant temperature room for the 8 week experimental period. Colonies were removed and drawn using a camera lucida at weekly intervals, the drawings later being digitized to obtain measurements of area and perimeter. Median proximal spine lengths were measured again at the end of the experiment, using the same methods as when originally examined. Sexually active zooids were also counted at the end of the experiment, using a Leitz Diavert inverted microscope. Total zooid number was tabulated at the end of the experiment.

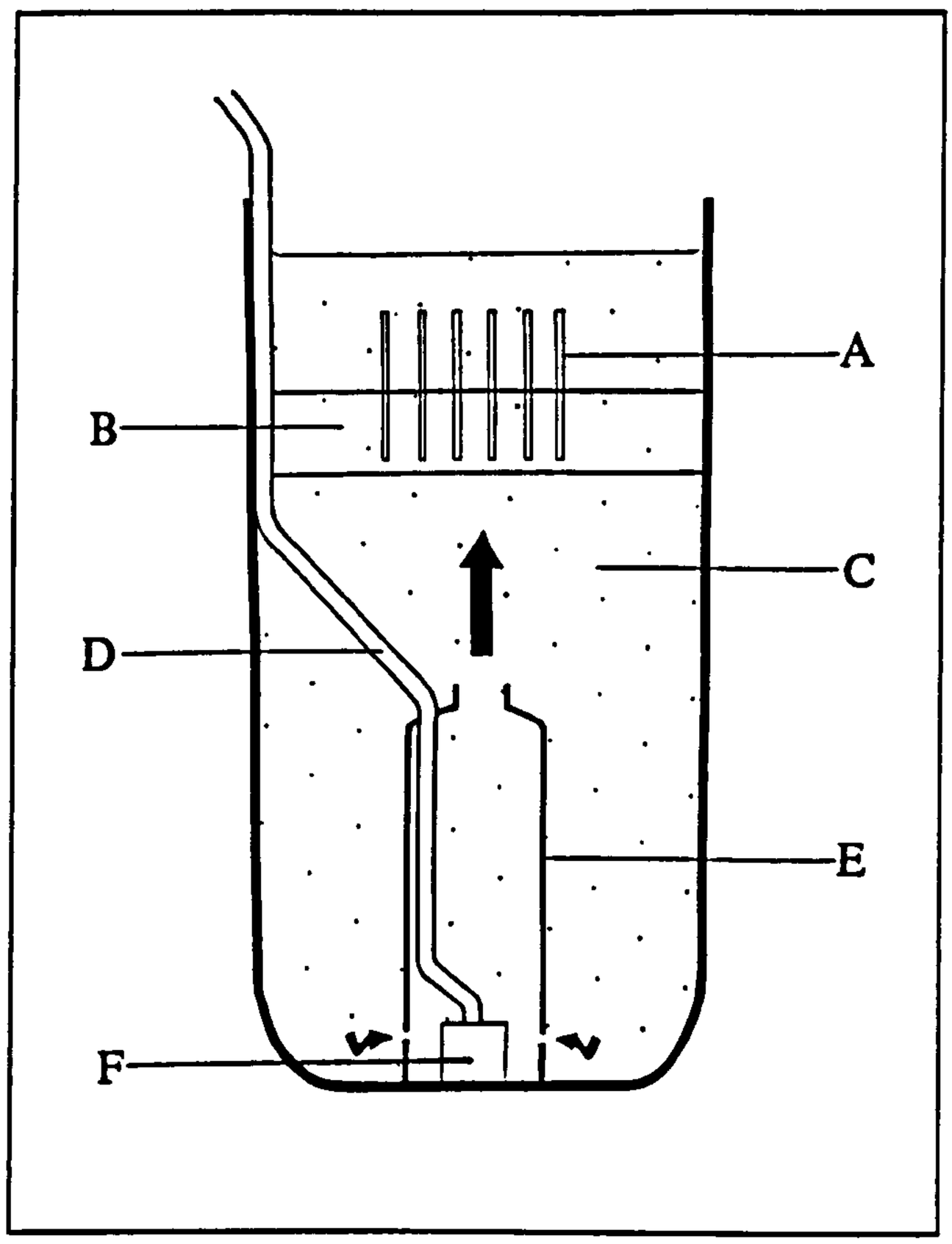


Figure 4.4. Diagram of apparatus used in turbulent flow experiment.

A = Glass slides supporting bryozoan colonies
B = Plastic slide rack
C = Sea water and algal suspension
D = Air supply
E = Syringe barrel
F = Airstone

Arrows indicate general direction of water movement.

4.2.3. Abrasion

Two experiments investigating the effects of abrasion were undertaken. Both experiments utilised 2 perspex cylinders (9 cm dia. x 31 cm length), mounted side by side on pivoting platform (Fig. 4.5). A sea-saw motion was achieved by the action of a rotating cam (34 rpm) working against elasticated cords attached to either end of the platform and base. A rise and fall of 11 cm was achieved at each end of the platform. Cylinders contained 1300 ml of 0.2 µm filtered, UV-irradiated seawater and algal suspension, 75 % *R. reticulata* and 25 % *I. galbana* (100 cells μl^{-1}). The

water level was maintained in the cylinders, so that a gap in each allowed maximal water movement without uncovering colonies contained within. Each cylinder contained 3 plastic slide racks (78 mm x 80 mm x 21 mm) holding a total of 18 colonies on glass microscope slides (7.5 cm x 3.9 cm). Colonies were composed of 6 genotypes, 3 from Hell's Mouth (HM) and 3 from Church Island (CI). Three replicates of each genotype were placed in both cylinders. Slides were positioned parallel to the long axis of the cylinder within the racks. Aeration of the vessels was obtained via airstones positioned at one end of the cylinder. Air was allowed to escape from a tube situated vertically in the center of each cylinder. Seawater and algal suspension was changed daily. Maintenance and recording of colonies was performed as for the turbulent flow experiment (section 4.2.2). Two separate experiments were carried out using the apparatus described previously (Fig. 4.5). Genotypes used were also those used in the turbulence experiment (section 4.2.2).

1) Sand abrasion - To one cylinder 80 g of sterile sand (≈0.2 mm dia.) was added. Sand was renewed weekly. The second cylinder had no sand added, and was left as a control.

2) Plastic abrasion - Within one cylinder, sheets of plastic sheet (74 mm x 38 mm x 0.16 mm) were placed between each slide. One end of the plastic was glued to the slide rack, while the other was left free. The 'sloshing' motion of the apparatus caused the plastic to move against the surface of the colonies attached to the microscope slides. The second cylinder was left as a control with no plastic added.

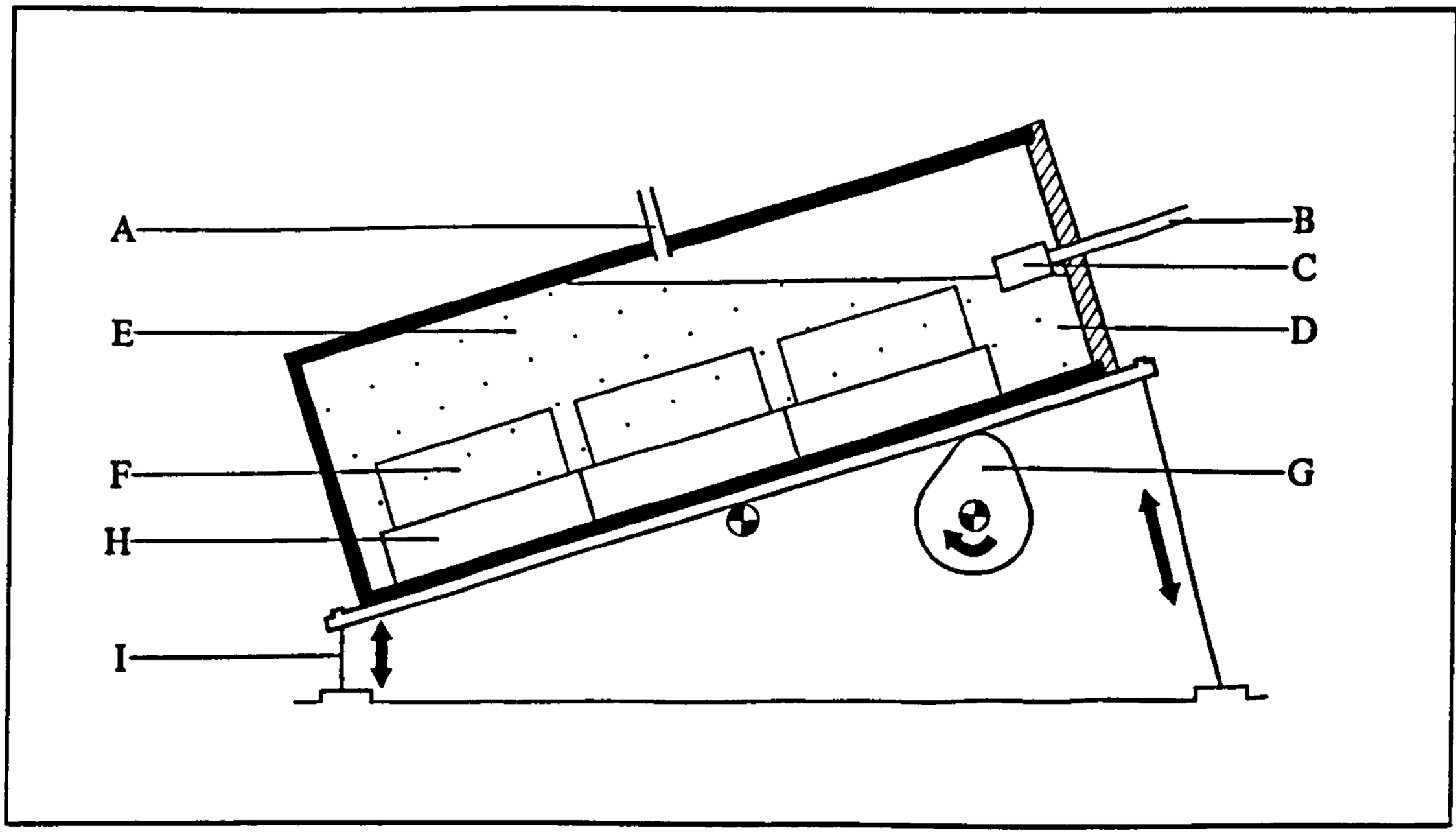


Figure 4.5. Diagram of apparatus used in abrasion experiments.

A = Breather pipe
B = Air supply
C = Airstone
D = Lid
E = Sea water and algal suspension
F = Slides supporting colonies
G = Cam
H = Plastic slide racks
I = Elasticated cord

Arrows indicate direction of movement.

4.2.4. Reciprocal transplants

Six rocks (≈80 cm dia.) with several fronds of *F. serratus* supporting healthy *E. pilosa* colonies were selected from both HM and CI. All other epiphytic organisms were removed from the algal fronds using a blunt scalpel. Bryozoan colonies were reduced to 40 zooids, 6-7 of which were at the growing edge of the colony. Colonies were then drawn on acetate sheets to mark their position on the fronds. Care was taken to select colonies at least 8 cm apart, to avoid any possible occurrence of overgrowth. The rocks were placed in the field on 1st August 1996 and retrieved 8 weeks later. Three rocks were returned to their original site accompanied by three from the opposite site. The rocks were marked using plastic ties and placed approximately 50 cm apart at the lower limit of the *F. serratus* zone. It was found necessary to secure rocks sited at HM, using stainless steel cable and rawl bolts, to prevent their loss. After 8 weeks colonies were drawn using a camera lucida. A total

of 20 median proximal spine lengths recorded for each colony, using a high powered Wild dissection microscope with eye-piece graticule.

4.2.5. Particle path measurement

Colonies of *E. pilosa* growing on *F. serratus*, were taken from HM and CI, this provided animals with contrasting long and short spines. Sections of *F. serratus* supporting healthy colonies were cut using a scalpel, to approximately 6 cm lengths and 1.4 cm widths and placed in a flume (Fig. 4.6). The flume contained 2.4 litres of 0.2 µm filtered, UV- irradiated seawater with *R. reticulata* added to obtain a concentration of approximately 3000 cells ml^{-1}. Water temperature was maintained at 18 °C by passing the water, via a coiled polythene pipe through a constant temperature bath (Grant W38). Particle paths and velocities were recorded using a video camera (Kappa CF 11/1) mounted on a Wild MC3 dissecting microscope. The microscope was positioned to view the colonies from the side. Video recording at 50 half-frames per second was undertaken using a Panasonic NV-FS200 HQ video recorder. Selected video images were copied by means of a video graphic printer (Sony UP-860 CE). Particle movements relative to colonies, were traced in successive frames and drawn on acetate sheets overlaying the video screen. Particle positions were plotted every 0.02 s, thus enabling trajectory and velocity for each particle to be calculated. Measurements were taken for spineous and non-spineous colonies.

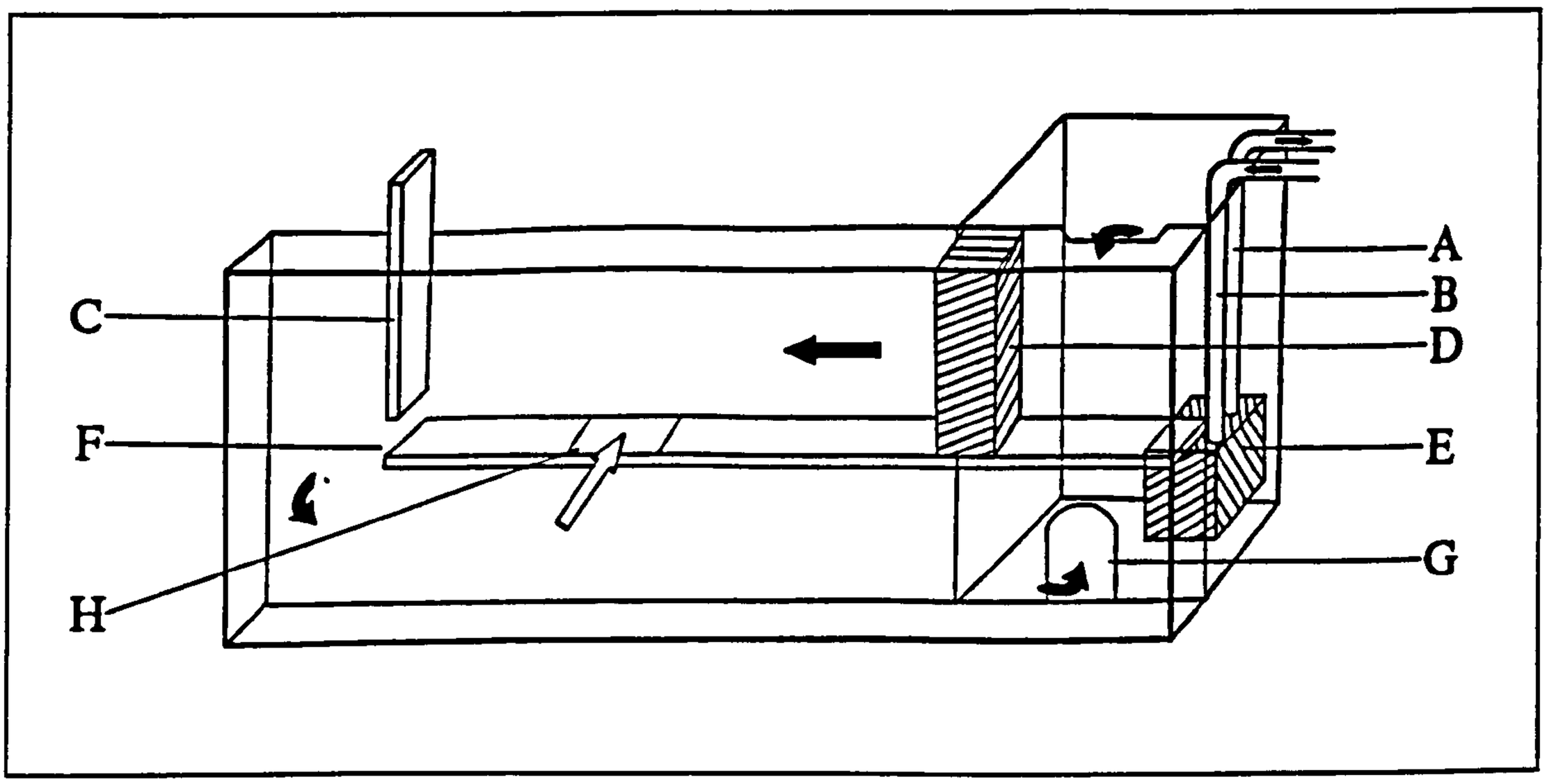


Figure 4.6. Flume used for particle trajectory measurements.

A = Water to water bath
B = Water from water bath
C = Adjustable door
D = Mineral wool baffle
E = Submersible pump
F = 5 mm gap
G = Intake port
H = Intake port Section of *F. serratus* supporting colonies

Shaded arrows indicate water direction. Un-shaded (white) arrow indicates direction of video camera.

4.2.6. Statistical analysis

Data obtained was subjected to the Anderson-Darling test to assess the normality of the data obtained, then analysed accordingly (Minitab, 1996).The general linear model (GLM) was applied unless data were found to be non-normally distributed, in which case Kruskal-Wallis test was applied (Zar, 1984). Multiple comparison test's were also undertaken where appropriate (Neave & Worthington, 1988). Particle velocity data were compared for colonies with short and long spines by using height above colony surface as a covariate in a GLM analysis of variance.

4.3. RESULTS

4.3.1. Natural spine length variation

4.3.1.1. Comparison of colonies from two contrasting study sites

Median proximal spine lengths were found to be significantly longer at HM than at CI (H=214.99, df=1, P=<0.001)(Fig 4.7 to 4.11). Zooid position from colony periphery was found to have a significant effect on spine length, both between sites (H=254.74, df=1, P=<0.001) and within sites CI (H=102.14, df=19, P=<0.001), HM (H=142.97, df=19, P=<0.001)(Fig. 4.11). Median proximal spine lengths increased away from the colony periphery (Fig 4.11 & 4.12). Number of additional opesial spines was observed to be higher at HM (8-10) than CI (2-4).

4.3.1.2. Degree of exposure

Percentage weight loss of the plaster of Paris spheres was greatest at CI(Z). Spheres located at HM showed greater weight loss than those located at CI(A) (Fig 4.13). Area CI(Z) supported 3 separate *F. serratus* plants in an area of less than 1 m^2. Approximately 15 colonies of *E. pilosa* with 'long' spines were found associated with these plants. All other colonies found at CI on *F. serratus* exhibited 'short' spines.

4.3.2. Turbulence

Spine lengths were found to be significantly different between colonies from CI and HM (H=538.88, df=1, P=<0.001) (Fig. 4.14). Colonies were analysed separately for each site. Spine lengths from HM colonies were significantly different between clones (H=6.93, df=2, P=0.032) but not between treatments (H=0.57, df=1, P=0.451). However, CI colonies showed no significant differences in spine lengths between clones (H=2.32, df=2, P=0.313) or between treatments (H=2.32, df=1, P=0.106).

In the light of these findings a further experiment was conducted one month later. Identical genotypes and conditions were utilised with the exception of an increase in the turbulence to 2000 ml air min^{-1}, the limit of the experimental equipment. Direct comparisons of data can not be made, due to the asynchrony of the

experiments. However, no colonies within the additional experiment produced 'long'(1000 μm) median proximal spines.

No significant difference in zooid number was found between either treatment (GLM. $F_{1,32}$=0.90 P=0.350), colony origin (GLM. $F_{1,32}$=3.34 P=0.077) or clone (GLM. $F_{5,24}$=1.68 P=0.177). However, treatment-origin interaction was statistically significant (GLM. $F_{1,32}$=4.79 P=0.036). Relative Colony Perimeter (RCP) (Appendix 1.1) was found to differ significantly between sites of colony origin (H=21.34, df=1, P= <0.001). RCP was greater for colonies from HM, indicating that these colonies possessed a more stellate form than those from CI. Number of sexually active zooids were found to be significantly greater in CI colonies than HM colonies (H=8.29, df=1, P=0.004). All colonies exceeded 100 zooids in size the limit for sexual maturity observed in field populations by Hughes (1986). Effect of treatment and clone were examined separately for each site (Tables 4.1 and 4.2).

Table 4.1. Kruskal-Wallis analysis of RCP and number of sexually active zooids in HM colonies of *E. pilosa*, exposed to turbulent flow.

Variable	Factor	H value	DF	P value
RCP	treatment	11.56	1	0.001*
	clone	1.82	2	0.402
No. SAZ	treatment	0.56	1	0.453
	clone	8.47	2	0.015*

Data presented are H-values, Degrees of Freedom (DF) and Probabilities (P value). Relative Colony Perimeter (RCP) and Number of Sexually Active Zooids (No. SAZ). *, significant values.

Table 4.2. Kruskal-Wallis analysis of RCP and number of sexually active zooids in CI colonies of *E. pilosa*, exposed to turbulent flow.

Variable	Factor	H value	DF	P value
RCP	treatment	0.33	1	0.566
	clone	3.89	2	0.143
No. SAZ	treatment	1.64	1	0.201
	clone	0.32	2	0.854

Data presented are H-values, Degrees of Freedom (DF) and Probabilities (P value). Relative Colony Perimeter (RCP) and Number of Sexually Active Zooids (No. SAZ). *, significant values.

Only HM colonies displayed significant differences in colony form, more stellate colonies occurring in the low flow treatment. Number of sexually active zooids varied significantly between clones from HM.

4.3.3. Abrasion

4.3.3.1. Sand abrasion

A significant difference in spine length was detected between HM and CI colonies (H=371.97, df=1, P=<0.001) (Fig. 4.15). Colonies were analysed separately for each site. Spine lengths for CI colonies were found to differ significantly between clones (H=23.95, df=2, P=<0.001) but not between treatments (H=3.88, df=1, P=0.06). Spine lengths for HM colonies were also different between clones (H=10.37, df=2, P=0.006), but no significant difference was detected in the control treatment (H=0.01, df=1, P=0.911). Number of zooids was not found to differ significantly between treatments (GLM. $F_{1,32}$=0.07 P=0.795), colony origins (GLM. $F_{1,32}$=1.15 P=0.292) and there was no significant treatment-origin interaction (GLM. $F_{1,32}$=0.02 P=0.879). Zooid number did, however, differ significantly between clones (GLM. $F_{5,24}$=63.48 P=<0.001) . Both RCP values and number of sexually active zooids were significantly different between colony origin, wherein colonies from HM were more stellate than CI (H=11.68, df=1, P=0.001). Colonies from CI produced more sexually active zooids than those from HM (H=7.23, df=1, P=0.007). Effect of treatment and clone on both RCP and number of sexually active zooids was examined separately for each site (Tables 4.3 and 4.4).

Table 4.3. Kruskal-Wallis analysis of RCP and number of sexually active zooids in HM colonies of *E. pilosa*, exposed to sand abrasion.

Variable	Factor	H value	DF	P value
RCP	treatment	0.33	1	0.566
	clone	15.16	2	0.001*
No. SAZ	treatment	1.32	1	0.251
	clone	8.13	2	0.017*

Data presented are H-values, Degrees of Freedom (DF) and Probabilities (P value).
Relative Colony Perimeter (RCP) and Number of Sexually Active Zooids (No. SAZ) *, significant values.

Table 4.4. Kruskal-Wallis analysis of RCP and Number of sexually active zooids in CI colonies of *E. pilosa*, exposed to sand abrasion.

Variable	Factor	H value	DF	P value
RCP	treatment	1.64	1	0.201
	clone	3.19	2	0.203
No. SAZ	treatment	5.07	1	0.025*
	clone	4.22	2	0.122

Data presented are H-values, Degrees of Freedom (DF) and Probabilities (P value).
Relative Colony Perimeter (RCP) and Number of Sexually Active Zooids (No. SAZ) *, significant values.

HM colonies exhibited significant variation amongst clones both for number of sexually active zooids and colony form. CI colonies however, differed significantly only in the number of sexually active zooids between treatments, the control treatment having colonies with more sexually active zooids than the colonies exposed to sand abrasion.

4.3.3.2. Plastic abrasion

A significant difference in spine length was detected between HM and CI colonies (H=539.25, df=1, P=<0.001) (Fig. 4.16). Differences in spine lengths between clones were significant for both CI and HM colonies (H=27.90, df=2, P=<0.001 and H=118.03, df=2, P=<0.001, respectively). However, no significant spine length differences were found between treatments for either CI (H=0.32, df=1, P=0.571) or HM colonies (H=0.92, df=1, P=0.337). Although, no overall significant variation was detected between treatments, two HM colonies did produce single zooids with 'long' spines under the abrasive treatment. Number of zooids did not differ significantly between treatments (GLM. $F_{1,32}$=0.06 P=0.803), colony origins (GLM. $F_{1,32}$=0.00 P=0.956) or treatment-origin interaction (GLM. $F_{1,32}$=0.06 P=0.803). A significant difference in zooid number was detected between clones (GLM. $F_{5,24}$=8.64 P=<0.001). Number of sexually active zooids and RCP values were significantly different between colony origins (H=8.66, df=1, P=0.002 and H=8.47, df=1, P=0.004, respectively). Data were consequently split between

localities to examine treatment and clonal responses of RCP and number of sexually active zooids (Table 4.5 and 4.6).

Table 4.5. Kruskal-Wallis analysis of RCP and number of sexually active zooids in HM colonies of *E. pilosa*, exposed to abrasion by a local surface.

Variable	Factor	H value	DF	P value
RCP	treatment	8.24	1	0.004*
	clone	0.11	2	0.949
No. SAZ	treatment	0.70	1	0.402
	clone	5.05	2	0.080

Data presented are H-values, Degrees of Freedom (DF) and Probabilities (P value). Relative Colony Perimeter (RCP) and Number of Sexually Active Zooids (No. SAZ).*, significant values.

Table 4.6. Kruskal-Wallis analysis of RCP and number of sexually active zooids in CI colonies of *E. pilosa*, exposed to abrasion by a local surface.

Variable	Factor	H value	DF	P value
RCP	treatment	0.05	1	0.825
	clone	2.05	2	0.360
No. SAZ	treatment	3.60	1	0.058
	clone	2.12	2	0.347

Data presented are H-values, Degrees of Freedom (DF) and Probabilities (P value). Relative Colony Perimeter (RCP) and Number of Sexually Active Zooids (No. SAZ). *, significant values.

RCP values for HM colonies indicate a significant difference in colony form between treatments, colonies exposed to abrasion having a more compact form than control colonies.

4.3.4. Reciprocal transplants

Spine lengths were found to be significantly different between all the groups of colonies, both reciprocal transplants and control colonies (Table 4.7) (Fig 4.17).

Table 4.7. Dunn's multiple comparison test of spine length variation in reciprocally transplanted colonies.

Location and Movement			Mean Difference	P value
CI to HM	vs	CI to CI	1025.40	<0.001*
CI to HM	vs	HM to CI	337.32	<0.001*
CI to HM	vs	HM to HM	-198.29	<0.001*
CI to CI	vs	HM to CI	-688.04	<0.001*
CI to CI	vs	HM to HM	-1223.70	<0.001*
HM to CI	vs	HM to HM	-535.61	<0.001*

Data presented are Probabilities (P value). *, significant values. (n=499).

Significant differences in colony area were detected between most groups. However, no significant difference was observed either between colonies transplanted to HM and the CI controls colonies, or between the two groups which were moved to opposite sites. Control colonies were generally larger than transplants (Table 4.8) (Fig. 4.18.).

Table 4.8. Dunn's multiple comparison test of area variation in reciprocally transplanted colonies.

Location and Movement			Mean Difference	P value
CI to HM	vs	CI to CI	-43.221	>0.05
CI to HM	vs	HM to CI	-10.860	>0.05
CI to HM	vs	HM to HM	-98.155	<0.001*
CI to CI	vs	HM to CI	32.362	>0.05
CI to CI	vs	HM to HM	-54.934	<0.05 *
HM to CI	vs	HM to HM	-87.296	<0.001*

Data presented are Probabilities (P value). *, significant values. CI to HM (n=40), CI to CI (n=63), HM to CI (n=186), HM to HM (n=92).

Significant differences in RCP values were observed between most groups, although, comparisons between transplantees and controls from the site of transplantation were not found to differ significantly (Table 4.9)(Fig. 4.19). Colonies transplanted or returned to HM had a significantly less circular form than those from CI.

Table 4.9. Dunn's multiple comparison test of RCP variation in reciprocally transplanted colonies.

Location and Movement			Mean Difference	P value
CI to HM	vs	CI to CI	139.77	<0.001*
CI to HM	vs	HM to CI	116.89	<0.001*
CI to HM	vs	HM to HM	14.80	>0.05
CI to CI	vs	HM to CI	-22.87	>0.05
CI to CI	vs	HM to HM	-124.96	<0.001*
HM to CI	vs	HM to HM	-102.09	<0.001*

Data presented are Probabilities (P value). *, significant values. CI to HM (n=40), CI to CI (n=63), HM to CI (n=186), HM to HM (n=92).

Colony survivorship was highest in those groups returned to their original site. Colonies returned to CI had the lowest mortality (22 %). Colonies transplanted to CI from HM suffered the lowest survivorship, with loss of 50 % of the colonies (Fig. 4.20). Despite bolting the rocks supporting *F. serratus* fronds to the substratum at HM one rock was lost during the experiment, resulting in reduced numbers of CI to HM transplants (from 147 colonies to 78 colonies).

4.3.5. Particle path measurement

Particle velocities were analysed in the presence and absence of spines, using height as a covariate. Significant differences were detected for the spine-height interaction (GLM. $F_{1,11}$=102.3 P=<0.001) indicating the regression coefficients of 'long' and 'short' spined colonies were statistically distinct. Particles were found to travel considerably more slowly once beneath the region of 'long' spines, than when traveling over 'short' spined colonies (Fig. 4.21 & 4.22). Extended lophophores clearly operate within the region of spine influence on flow velocity (Fig. 4.9).

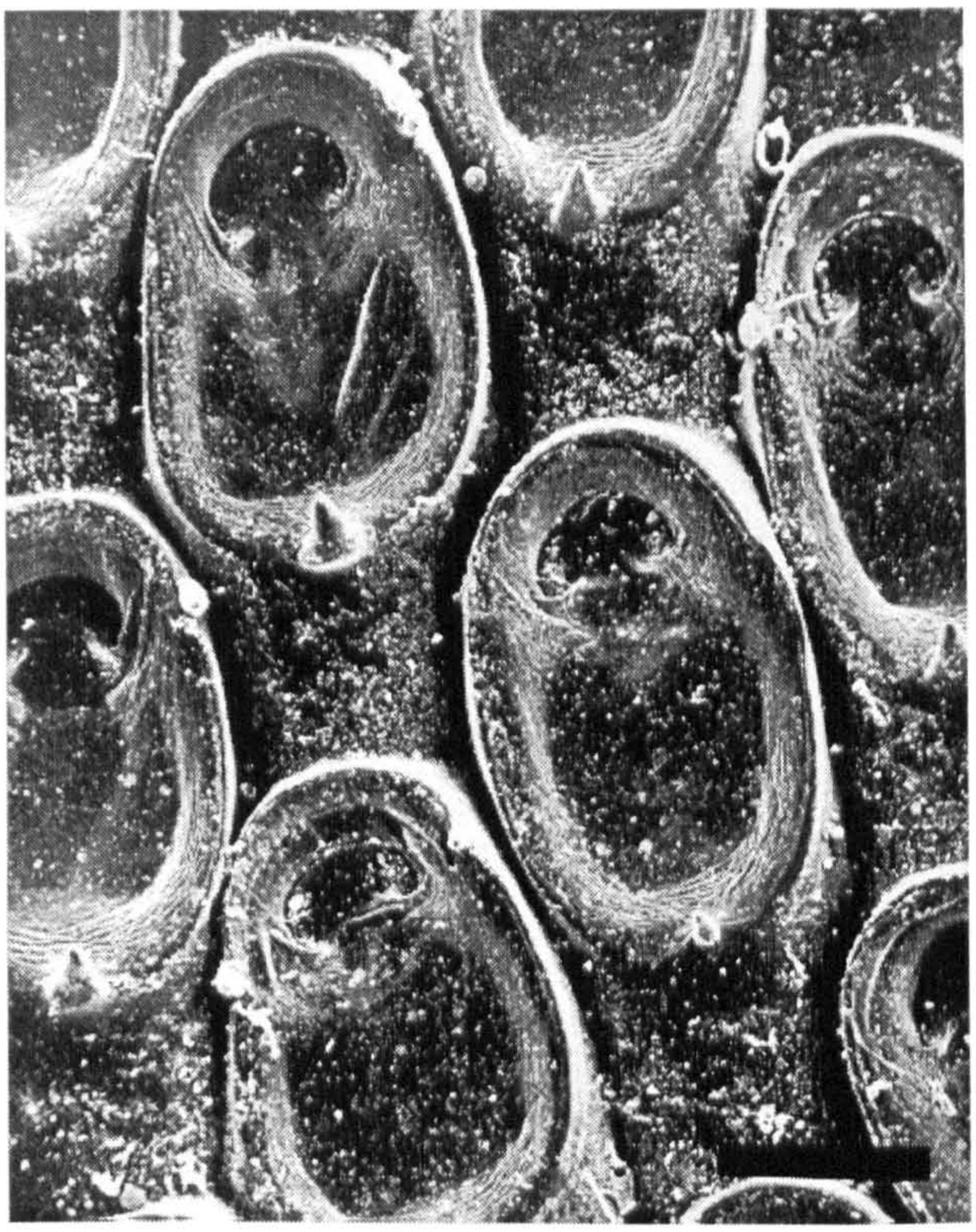


Figure 4.7. Scanning electron micrograph of *E. pilosa* zooids from CI exhibiting 'short' median proximal spines. Scale bar = 200 μm.

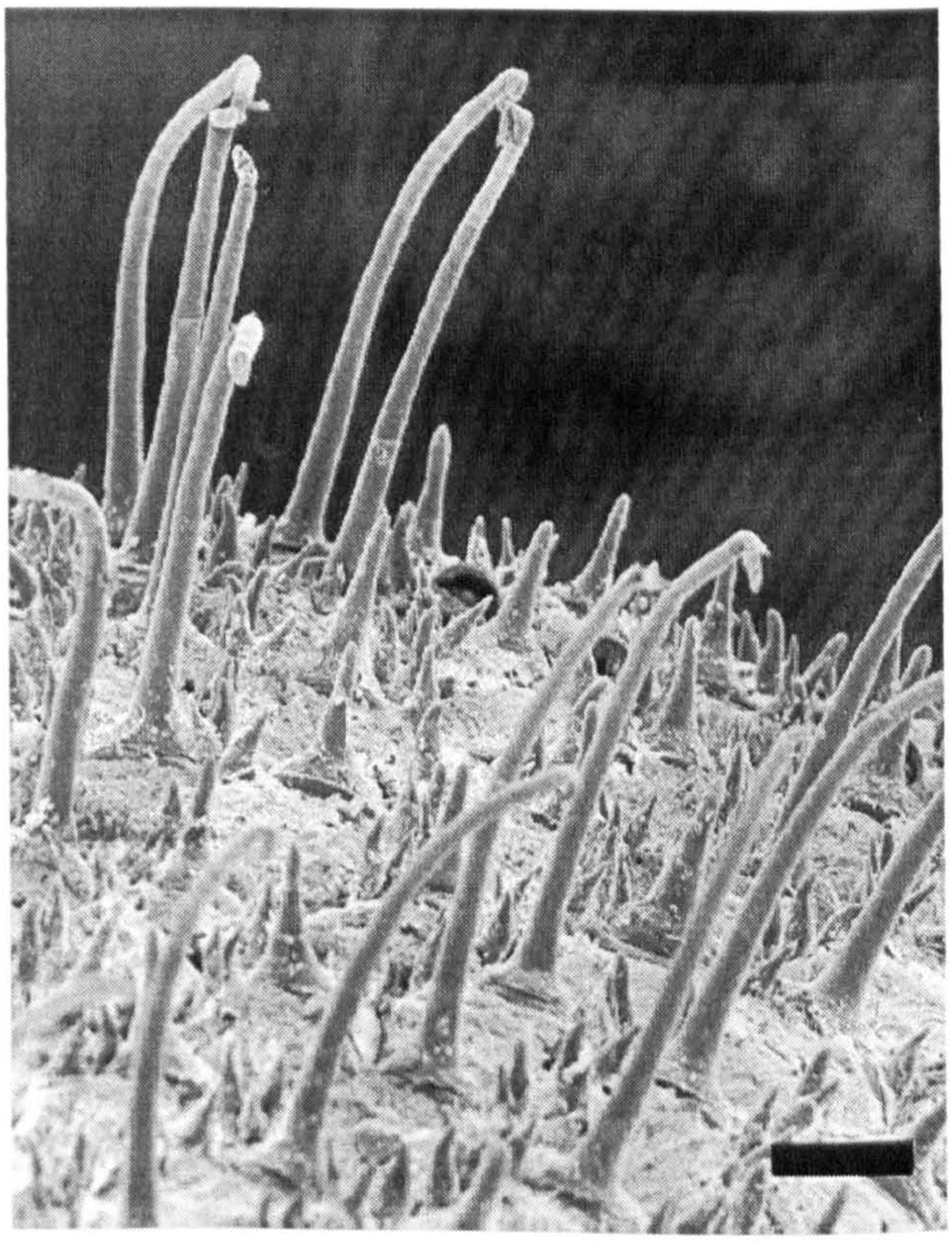


Figure 4.8. Scanning electron micrograph of a HM colony exhibiting zooids with 'long' median proximal spines. Scale bar = 200 μm.

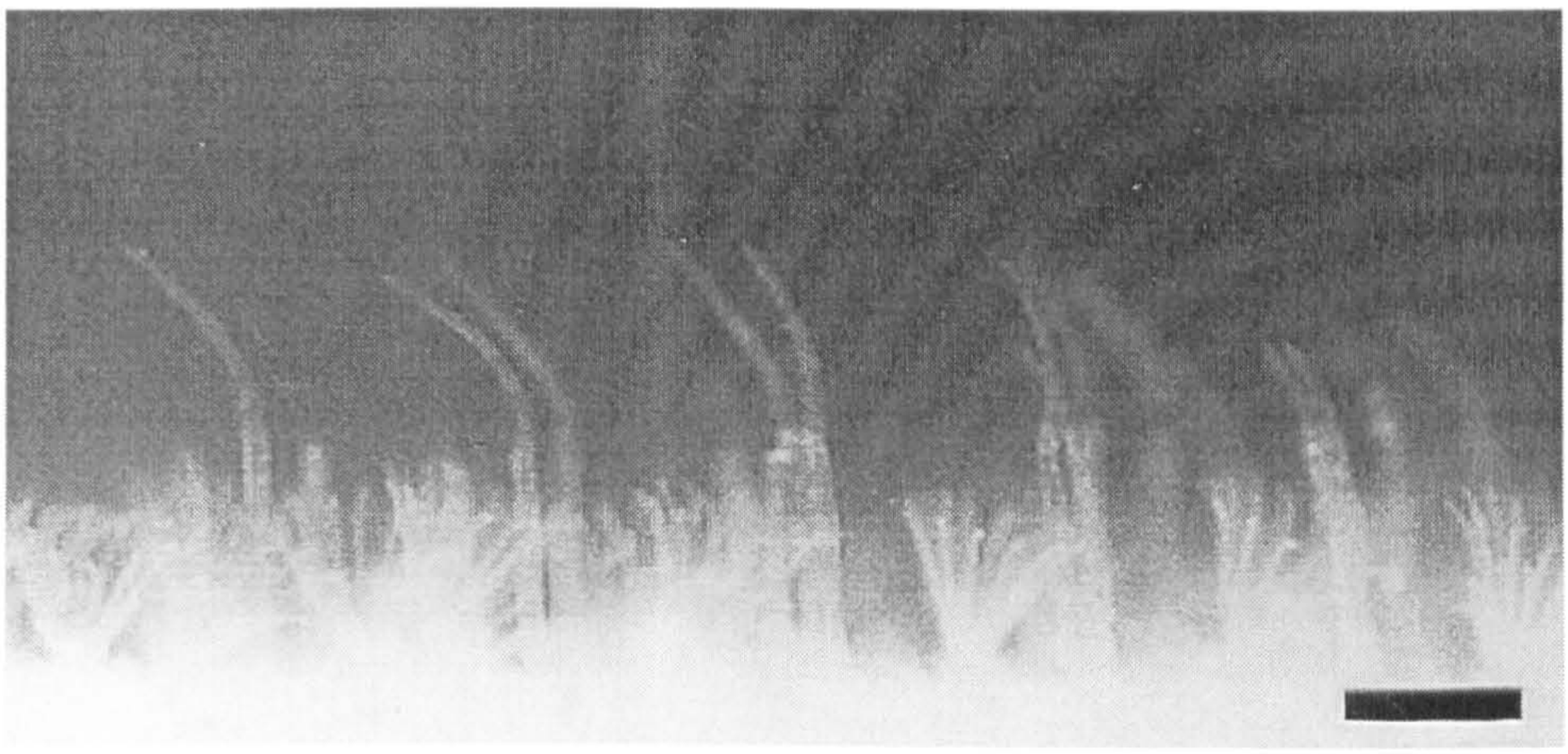


Figure 4.9. Picture of a 'long' spined colony with extended lophophores. Scale bar = 400 μm.

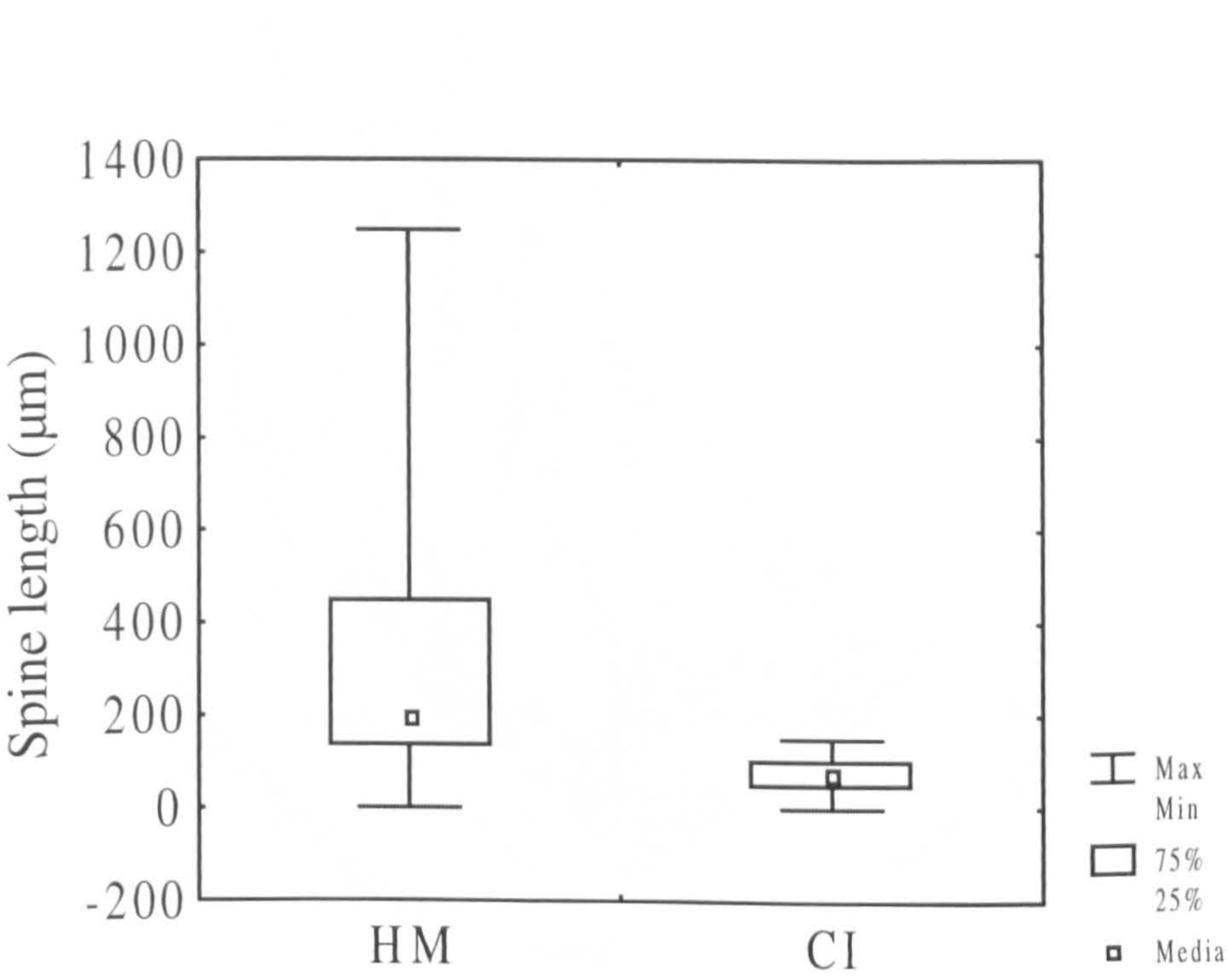


Figure 4.10. Spine lengths (μm) of *E. pilosa* colonies collected from both CI and HM. (n = 500). 75% to 25% boxes define upper and lower quartiles.

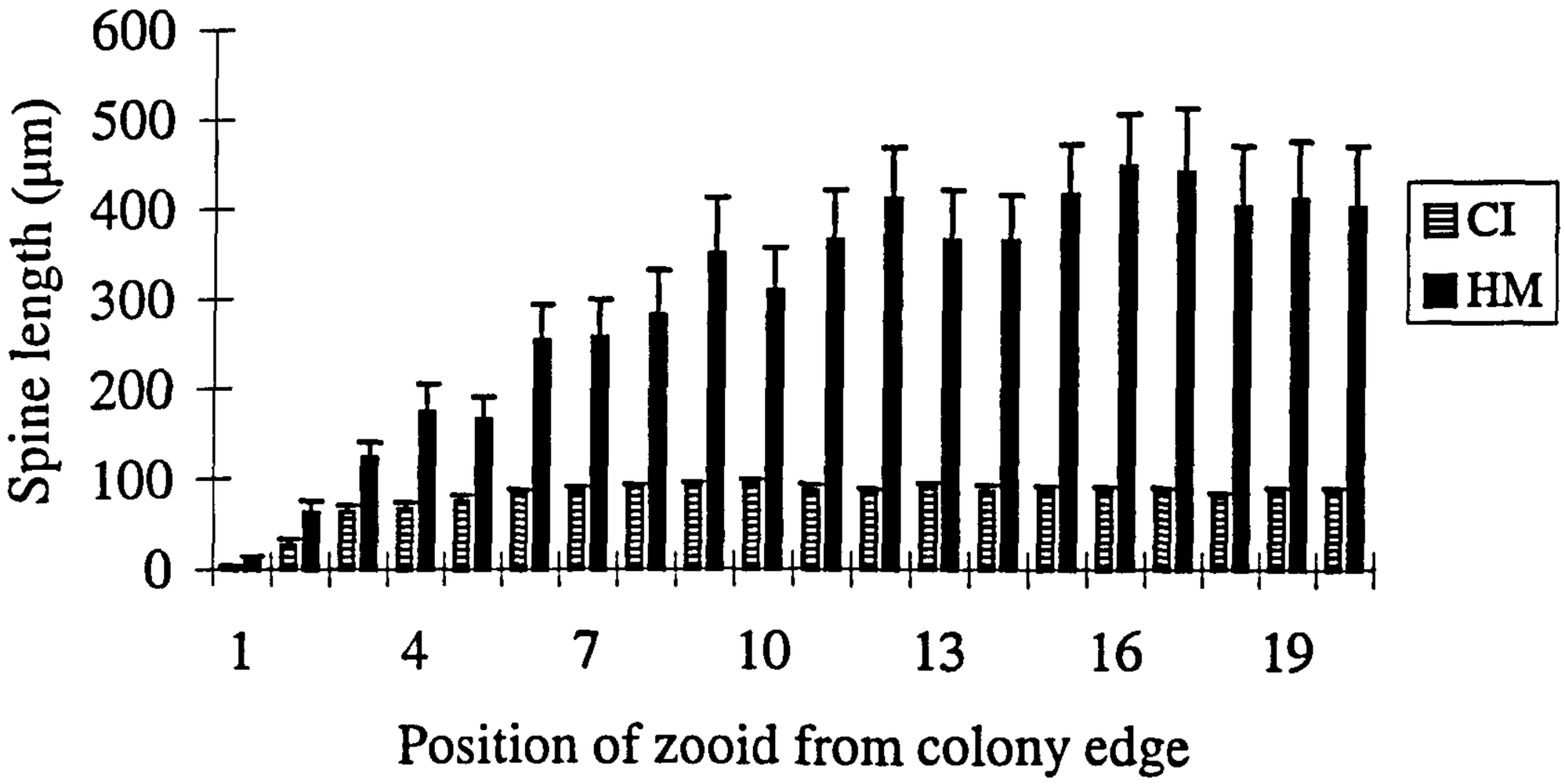


Figure 4.11. Length of median proximal spine (µm)(Mean + 1SE), for successive zooids from the colony edge for colonies of *E. pilosa* from both CI and HM (n =200).

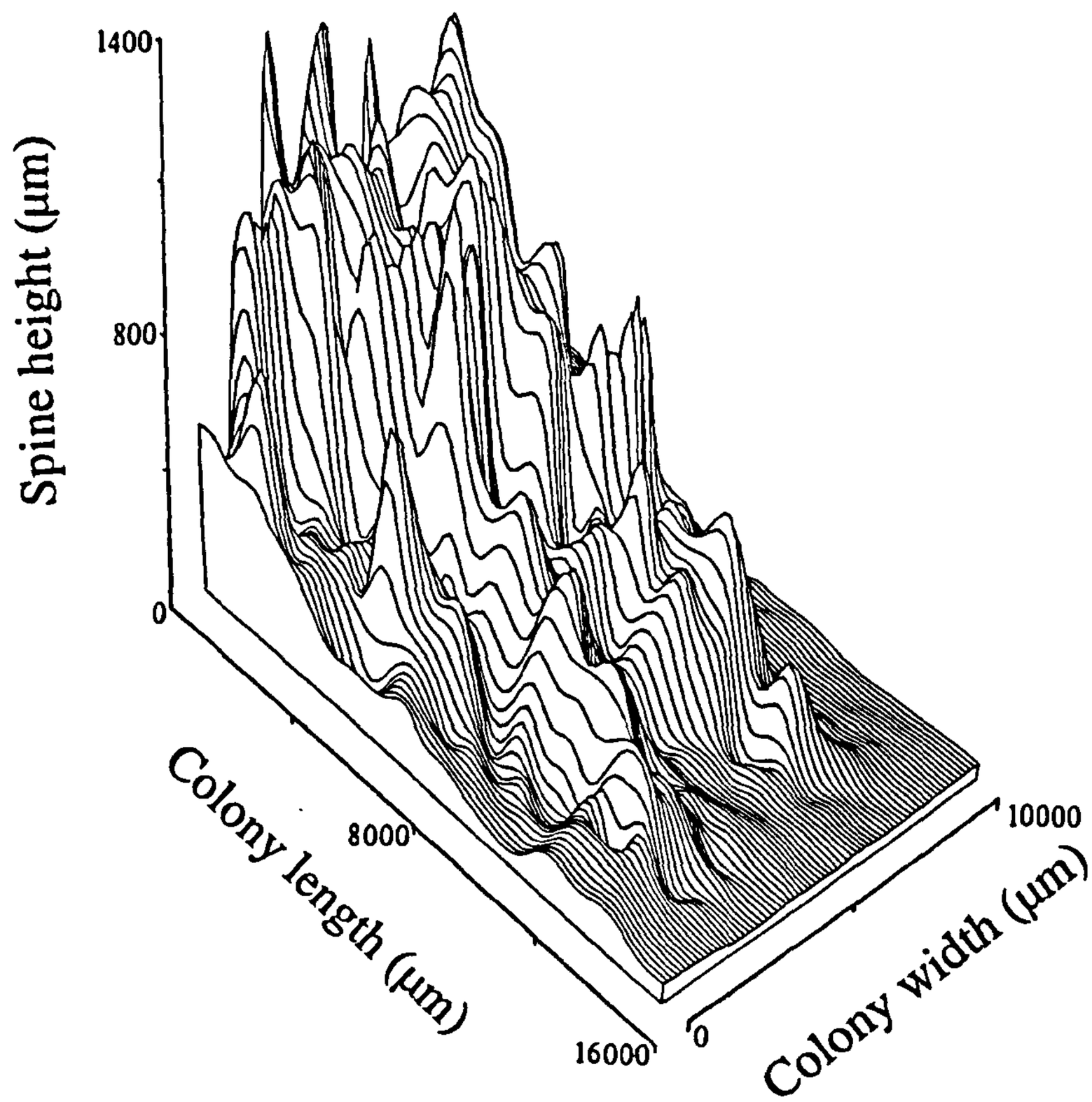


Figure 4.12. Three dimensional diagrammatic representation of spine lengths in a colony of *E.pilosa* (HM).

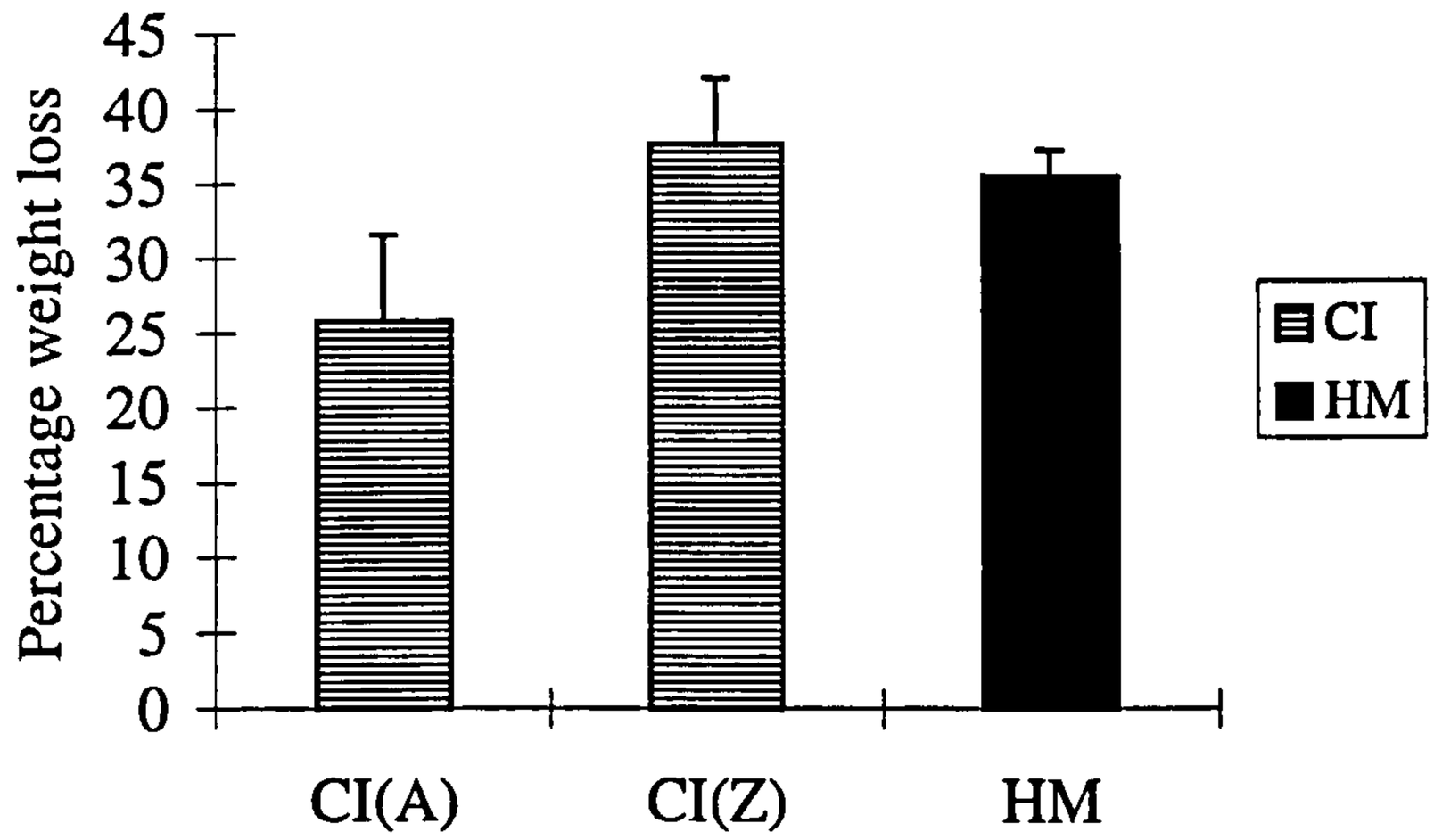


Figure 4.13. Percentage weight loss from gypsum spheres placed at 2 sites at CI and a single site at HM, positioned in the lower portion of the *F. serratus* zone. (n=3)

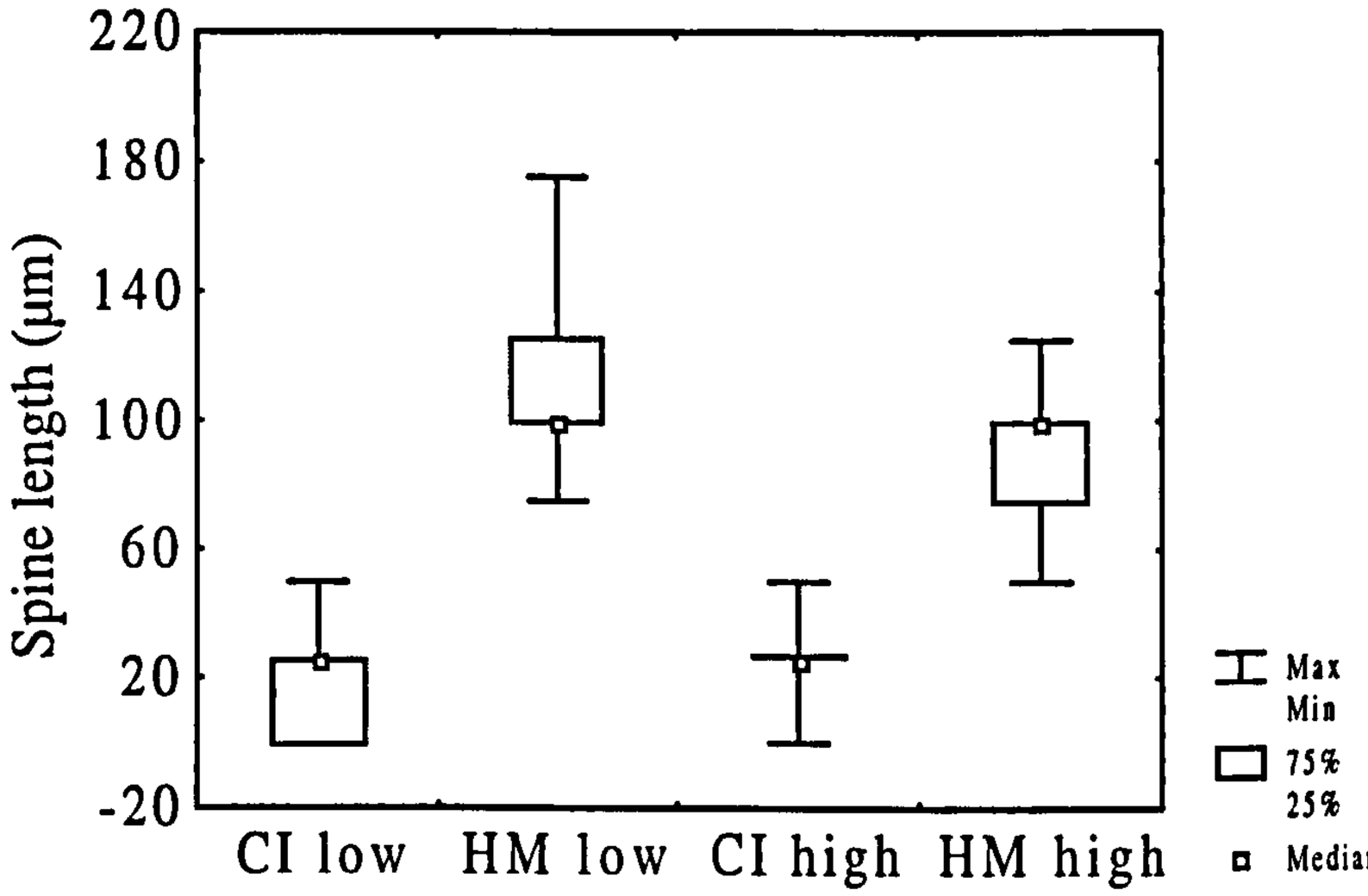


Figure 4.14. Spine lengths (µm) of colonies of *E. pilosa* from both CI and HM subjected to either, low (50 ml air min^{-1}) or high (1500 ml air min^{-1}) turbulence. (Colonies n=9, Genotypes n=3). 75% to 25% boxes define upper and lower quartiles.

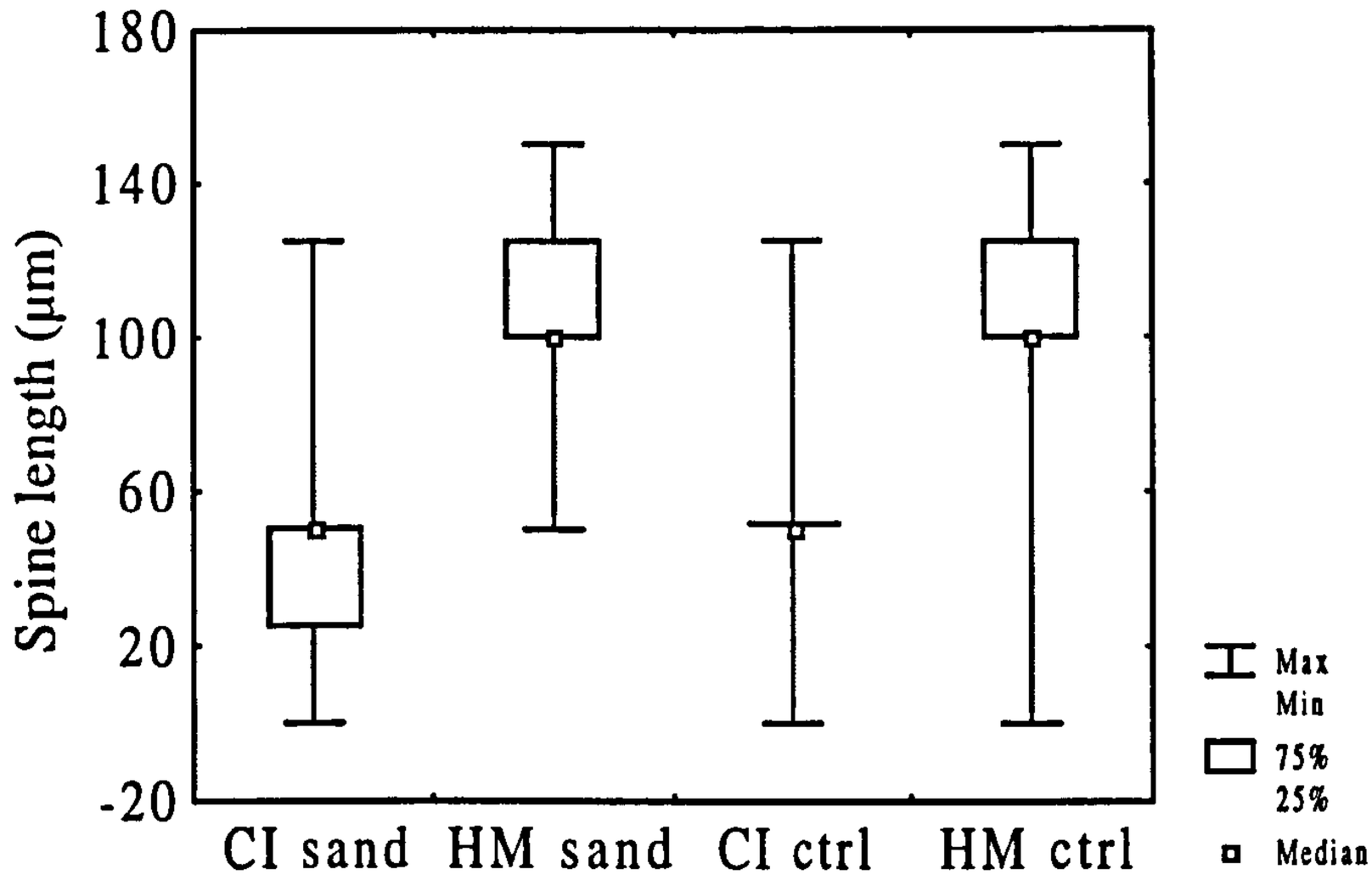


Figure 4.15. Spine lengths (µm) of colonies of *E. pilosa* from both CI and HM subjected to, either sand abrasion (sand) or no sand abrasion (ctrl). (Colonies n=9, Genotypes n=3). 75% to 25% boxes define upper and lower quartiles.

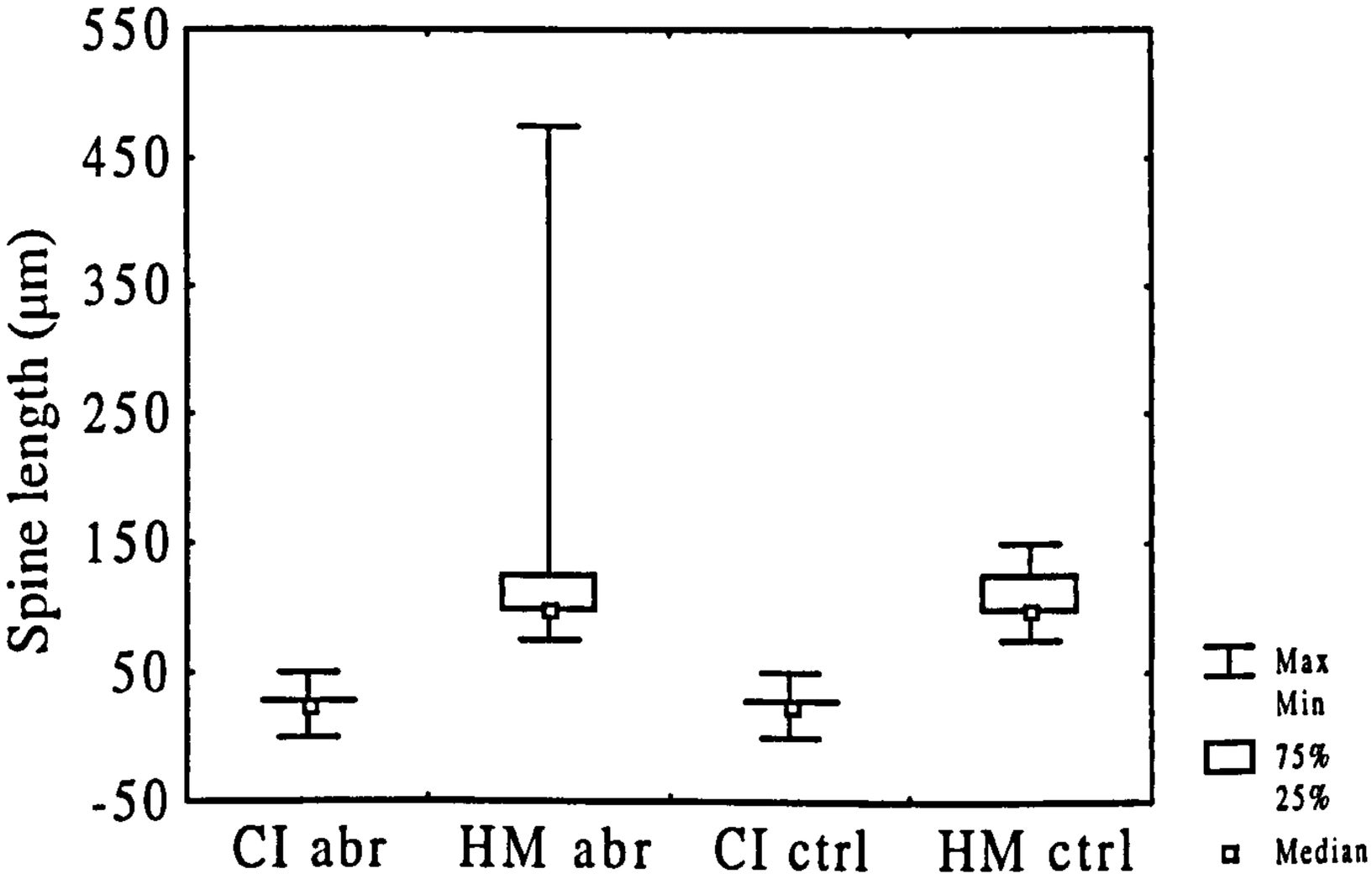


Figure 4.16. Spine lengths (µm) of colonies of *E. pilosa* from both CI and HM subjected to, either plastic abrasion (abr) or no plastic abrasion (ctrl). (Colonies n=9, Genotypes n=3). 75% to 25% boxes define upper and lower quartiles.

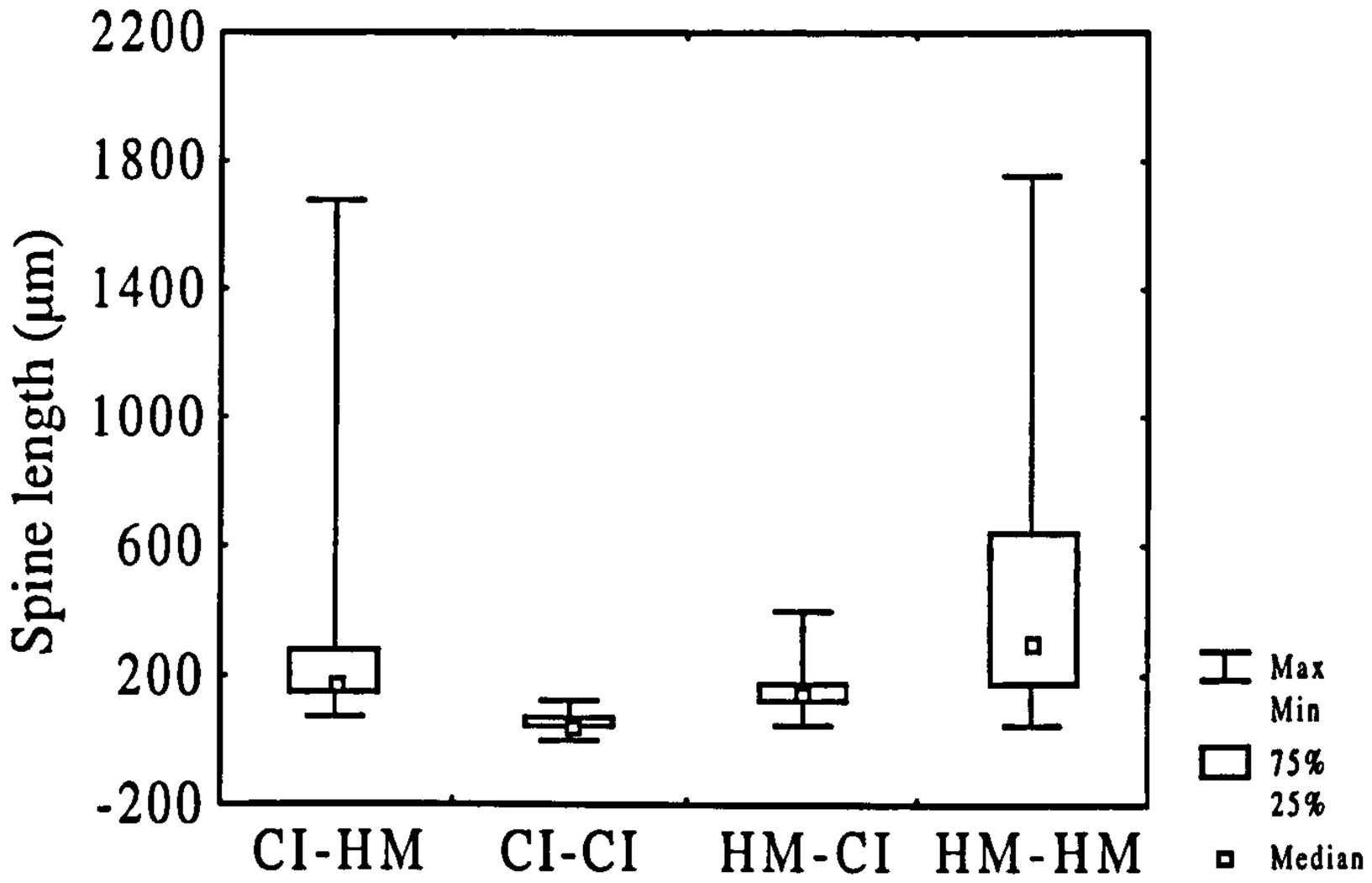


Figure 4.17. Spine lengths (µm) for reciprocally transplanted colonies. CI-HM = colonies moved from CI to HM; HM-CI = colonies moved from HM to CI; Colonies returned to their original site are shown as either HM-HM or CI-CI. (n=500). 75% to 25% boxes define upper and lower quartiles.

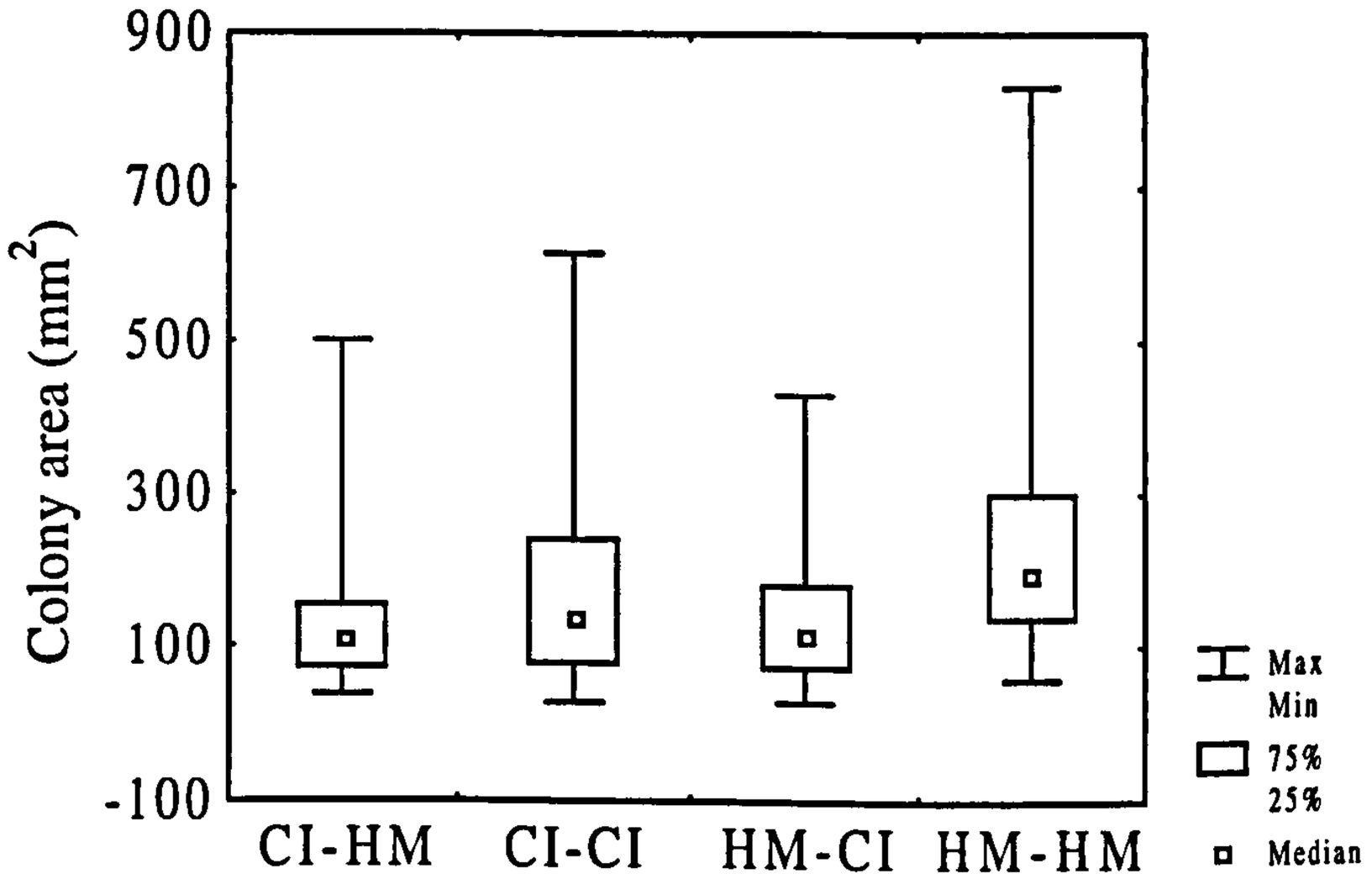


Figure 4.18. Colony area (mm^2) for reciprocally transplanted colonies at the end of the 8 weeks experimental period. CI-HM = colonies moved from CI to HM; HM-CI = colonies moved from HM to CI; Colonies returned to their original site are shown as either HM-HM or CI-CI. CI-HM (n=40), CI-CI (n=63), HM-CI (n=186), HM-HM (n=92). 75% to 25% boxes define upper and lower quartiles.

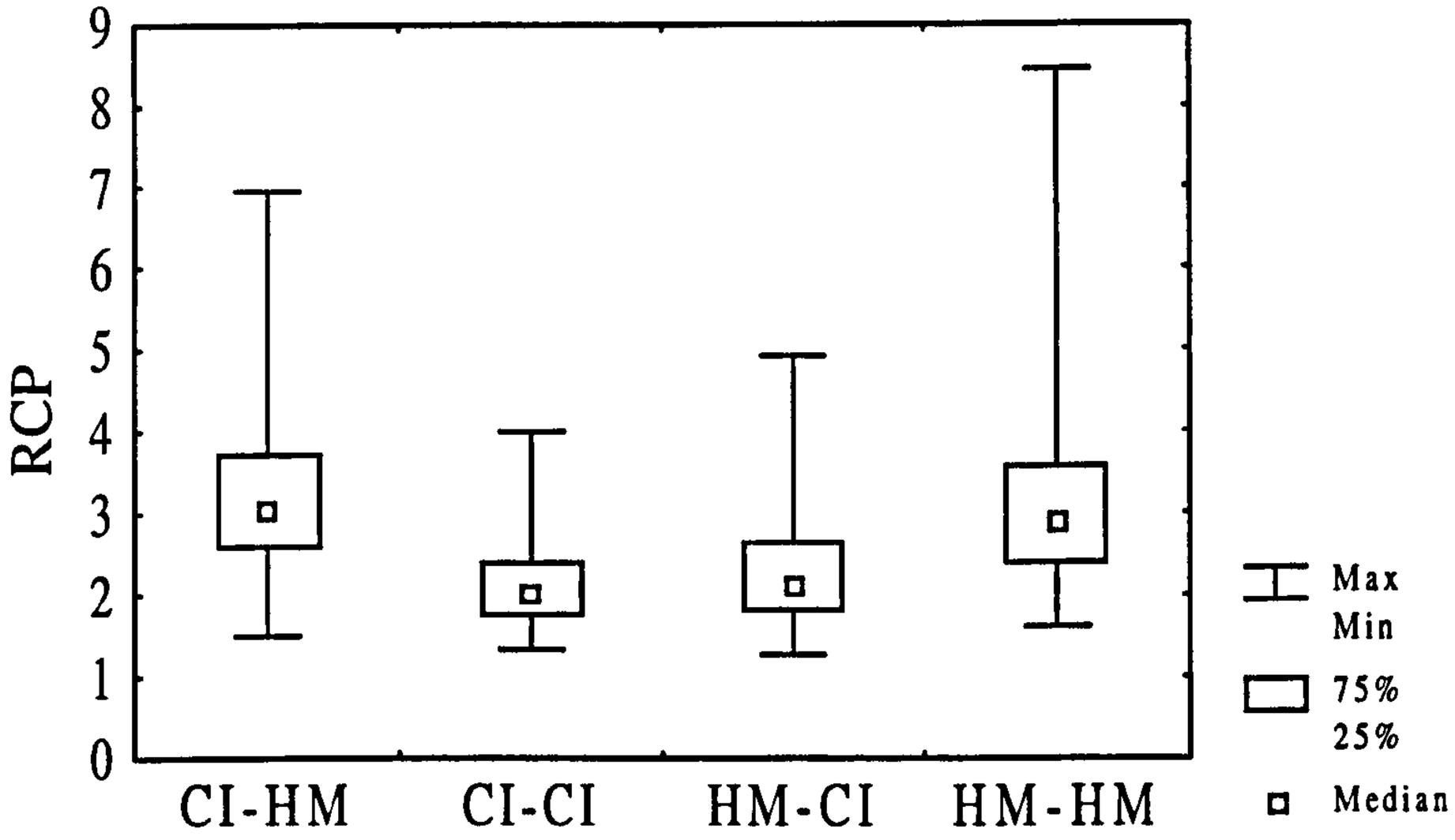


Figure 4.19. Relative Colony Perimeter (RCP) values for reciprocally transplanted colonies at the end of the 8 weeks experimental period. CI-HM = colonies moved from CI to HM; HM-CI = colonies moved from HM to CI; Colonies returned to their original site are shown as either HM-HM or CI-CI. CI-HM (n=40), CI-CI (n=63), HM-CI (n=186), HM-HM (n=92). 75% to 25% boxes define upper and lower quartiles.

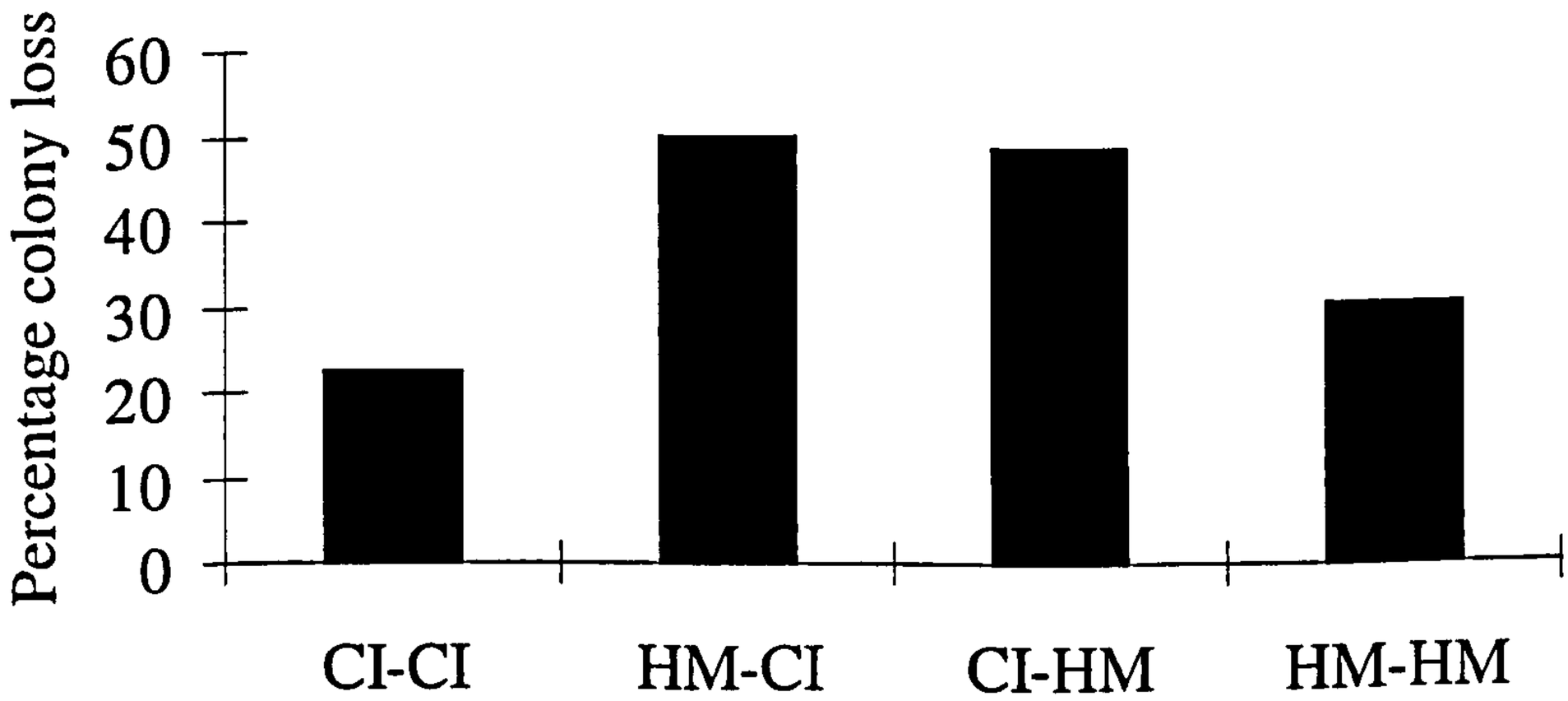


Figure 4.20. Percentage of colonies lost after the 8 weeks experimental period. CI-HM = colonies moved from CI to HM; HM-CI colonies moved from HM to CI; Colonies returned to their original site are shown as either HM-HM or CI-CI. CI-HM (n=40), CI-CI (n=63), HM-CI (n=186), HM-HM (n=92).

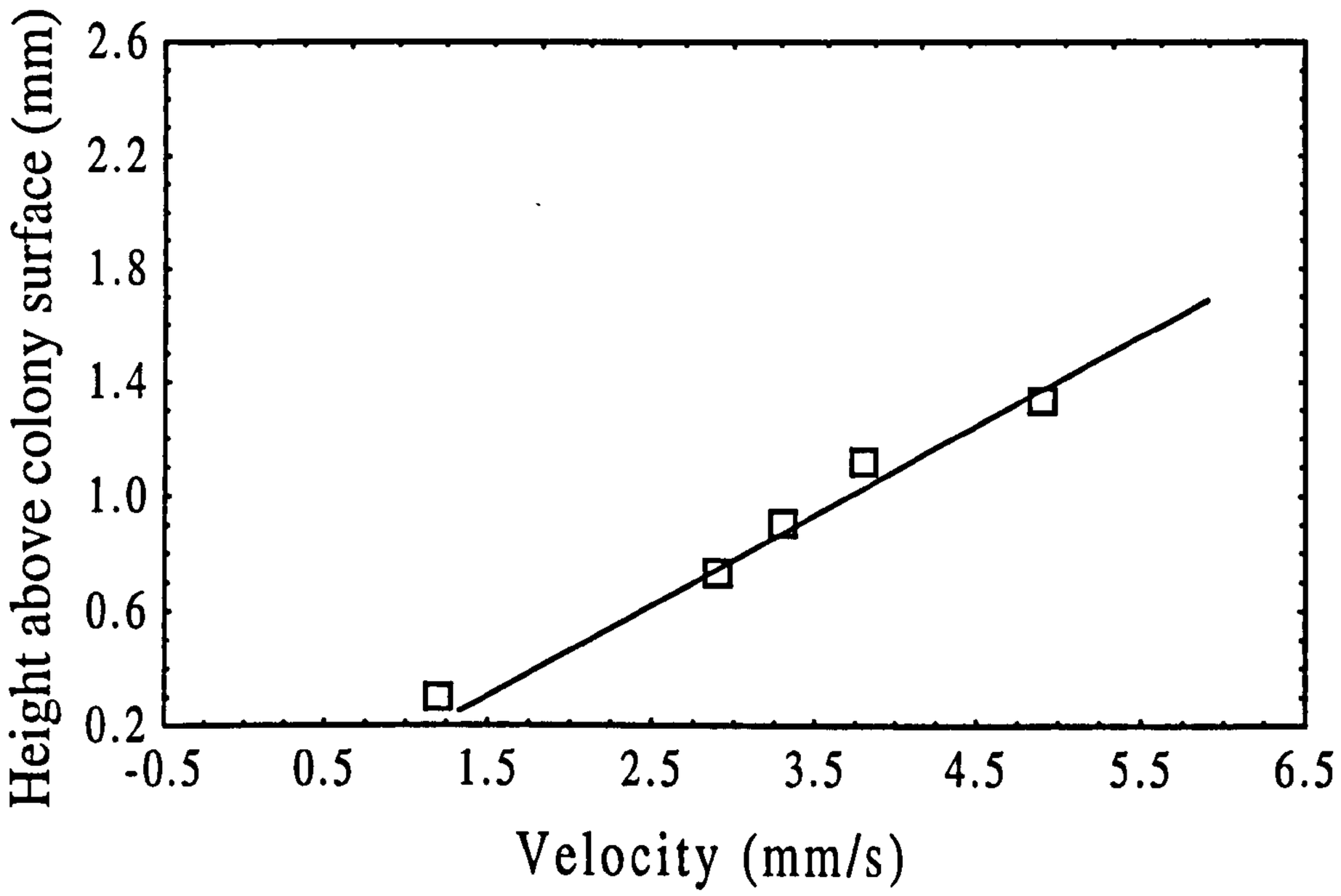


Figure 4.21. Relationship between algal (*R. reticulata*) particle velocity (mm/s) and height above 'short' spined *E. pilosa* colony surface (mm).

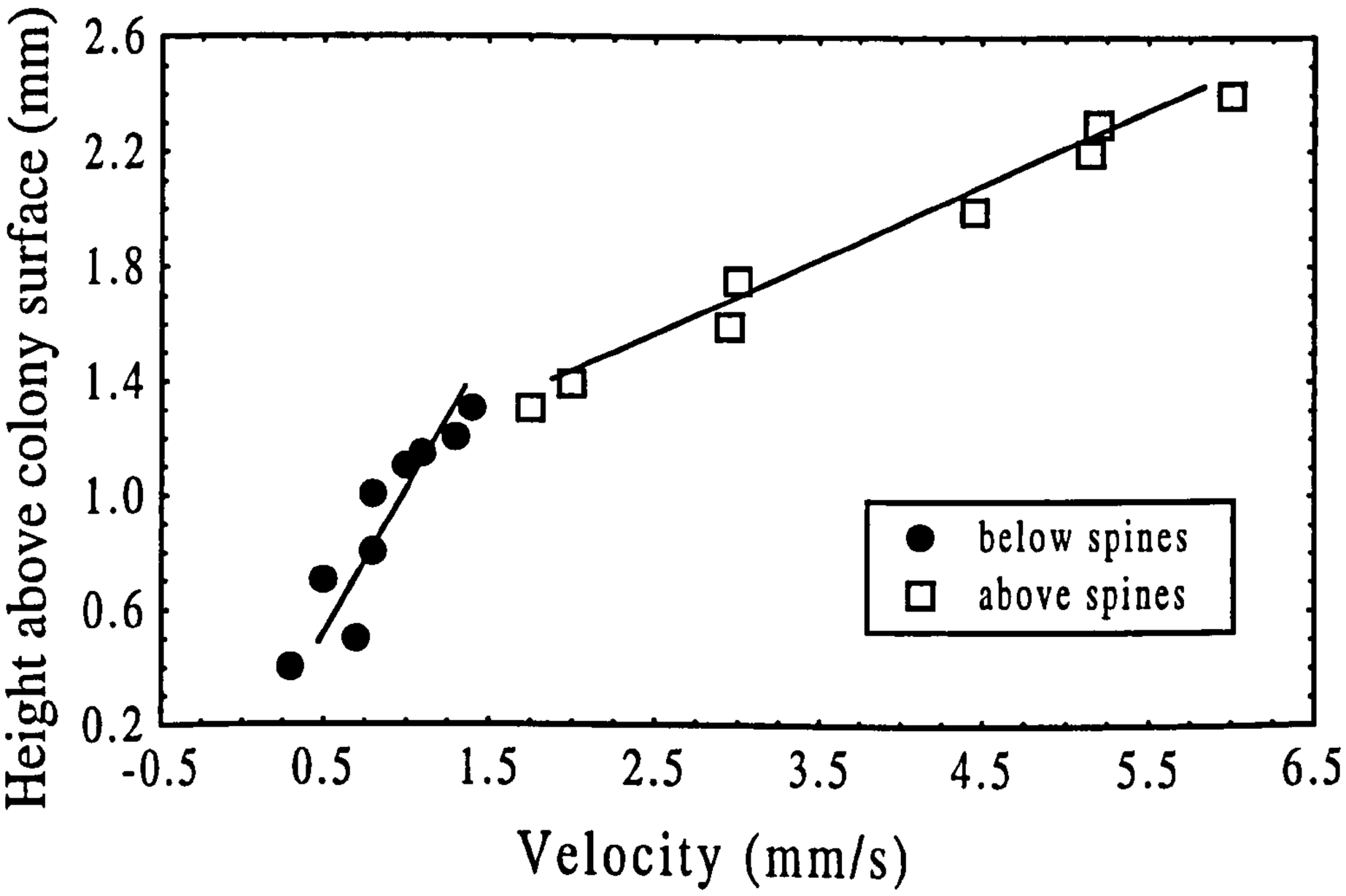


Figure 4.22. Relationship between algal (*R. reticulata*) particle velocity (mm/s) and height above 'long' spined *E. pilosa* colony surface (mm).

4.4. DISCUSSION

4.4.1. Field Observations

HM is the site of the highest exposure in terms of wave action. However, the largest weight loss from the calcium sulphate spheres was recorded at CI(Z). This can be explained by the fact that, although relatively more sheltered than HM, CI(Z) is the area of greatest overall water movement (Muus, 1968; Doty, 1971). Over a 24 hour period of relatively calm conditions, wave induced flow at HM is low in comparison to the large movement of water through the natural gully at CI(Z) during spring tides. The high degree of water movement at CI(Z) is likely to be associated with considerable turbulence and subsequently movement of algal fronds resulting in abrasion of colonies. Possibly, it is the combination of these forces present in the CI(Z) area which trigger the production of the 'long' spined colonies found.

With the exception of colonies at the CI(Z) site, *E. pilosa* colonies growing at the more exposed HM site produced longer median proximal spines than those at CI. All 'long' spined colonies showed an increase in the mean length of the median proximal spines away from the colony periphery. This is most likely due to the spines of the younger peripheral zooids needing time to achieve maximum length. However, spines fluctuated between extremes of length for each individual zooid, regardless of relative position. The polyphenic nature of the spine length plasticity is confirmed by the observed extremes in spine length between neighbouring zooids. It must therefore be assumed that each individual zooid is triggered separately to produce a 'long' median proximal spine which, in turn, provides the colony with the optimum spine coverage at minimum energetic outlay and lowest drag.

4.4.2. Laboratory Experiments

In all 3 of the laboratory experiments which individually investigated the effects of turbulence, sand abrasion and plastic abrasion on spine production, spine lengths were found to differ significantly between CI and HM colonies. These results suggest that there is a genetic component which controls residual spine length in *E. pilosa*.

With the exception of the production of 2 long spined zooids in the plastic abrasion treatment, no colonies in either test or control treatments for the 3

experiments produced 'long' spines. However, in all treatments, HM colonies exhibited significantly longer spines than those of the CI colonies. It is more than likely that colonies were not exposed to conditions of sufficient intensity to trigger changes in spine morphology.

4.4.3. Turbulence

Subjecting colonies to turbulence levels greater than those which initiated increased spine numbers in *F. hispida* had no observable effect on spine length (Whitehead *et al.*, 1996). The lack of variation in both the total number and sexually active zooids between the control and test treatments suggests that the colonies were subject to low stress levels. Variation in reproductive activity can be considered an indicator of stress in some bryozoans (Chapter 2). The HM colonies showed differences in number of sexually mature zooids between clones, highlighting the importance of genotype in the organisation of sexual parameters. This observation contradicts the findings of Bayer and Todd (1996), where no genetic contribution to sexual maturation in *E. pilosa* was established. Hunter & Hughes (1995) did, however, find significant genotypic contribution in sexual characteristics for *Celleporella hyalina*. Despite the lack of variation between treatments, the number of sexually mature zooids differed between colonies from the two sites examined, further demonstrating the disparity between the two populations (Chapters 2 & 3). CI colonies produced more sexually active zooids than HM colonies. It appears that CI colonies optimise energetic allocation to sexual production. This may be the result of selection for rapid sexual maturity in this population, due to intense competition and threat of overgrowth. However, such localised selection would be surprising given the widespread dispersal of the planktotrophic larvae of this species.

RCP proved to differ between populations, HM colonies being more stellate than CI colonies. Colonies from HM were found to be the most stellate in the control treatment. A stellate form has already been related to genotype (Bayer *et al.* 1994). However, within the HM population the variation between treatments could either be ascribed to rapid growth rate (Okamura, 1992; Bayer & Todd,1996) or a stressful growth environment (Winston, 1976; Jebram & Rummert, 1978). Previously, stellate or lobate form has been considered an indicator of stress in some bryozoan species (Hunter & Hughes, 1993b). Accordingly, ragged or stellate form may be attributed to

the development of uniserial 'runners' in *E. pilosa*, occurring under sub-optimal experimental conditions. Such uniserial chains of zooids are infrequent in nature, occasionally occurring in response to restricted substratum (Cook, 1977; Silén, 1987). In the present case, multiserial stellate growth, equality in zooid numbers and sexual activity between treatments, implies that stress was not the cause of stellate growth form. Consequently, the production of colonies deviating most from a circular configuration can be attributed to higher growth rate.

4.4.4. Sand abrasion

In the sand abrasion experiment, the only significant difference observed between treatments was within the CI population, which was found to produce more sexually active zooids in the control treatment. This implies, as might be expected, that a degree of stress was being created by the presence of sand particles. Suspended particulate material is thought to cause lophophore retraction as a preventative response to mechanical damage (Best & Thorpe, 1996). Such retractions will result in diminished feeding time and consequently reduced energetic acquisition for sexual allocation.

The majority of the remaining differences observed can be attributed to variations between colonies from the two populations. HM colonies were less circular in form than colonies from CI, mirroring the previous findings. In addition, CI colonies had a greater proportion of sexually active zooids than HM colonies. Finally, HM colonies exhibited significant variation between clones for both colony form and number of sexually active zooids, emphasising the importance of genotype in phenotypic response even within populations.

4.4.5. Plastic abrasion

The insignificant ' long' spine production of colonies subject to plastic abrasion is particularly disappointing in the light of the findings of other workers. Recently, *E. pilosa* colonies grown on fronds of *Fucus serratus*, subjected to artificial wave action, have been induced to yield colonies with a large proportion of 'long' spines (Bayer *et al.*, 1997). It must be concluded that in the present study, although attempts have been made to reproduce the degree of abrasion from neighbouring fronds found at wave swept localities, the degree of movement achieved was

insufficiently violent to initiate 'long' spine formation in the majority of zooids. However, two isolated zooids were observed to produce 'long' spines in the abrasion treatment.

No differences were detected between treatments, other than the formation of more stellate HM colonies in the controls. This is most likely an indication of a better growth environment and consequently a faster growth of colonies in the control group. CI colonies were again found to produce more sexually mature zooids and to be more circular in form than HM colonies. These factors are probably directly related, in that, a slower growth rate associated with more circular colony form is likely to be the result of energy being partitioned into reproductive activity thus reducing growth rate (Hughes, 1986).

4.4.6. Reciprocal transplants

Results from the reciprocal transplantation of colonies demonstrated that 'long' spine formation could be triggered in colonies from CI which had not previously displayed extended spines. Also, HM colonies exhibiting 'long' spines produced new zooids with 'short' spines once transplanted to CI. However, differences in spine lengths between colonies returned to their natal site and transplantees were detected. This variation between native and foreign colonies exposed to the same conditions implies a genetic component, highlighting the differences in the two populations. Although every effort was made to standardise the transplantation of colonies, some variation was inherent. Fronds of *F. serratus* varied in morphology between the two sites in question. Fucoids from highly exposed shores are known to have thinner thalli and are shorter lived than those from sheltered shores (Knight & Parke, 1950). High levels of turbulence have been found to result in retarded growth of *F. serratus* (Boaden *et al.*, 1975). Nevertheless, every effort was made to select plants of similar characteristics from both sites, but it remains possible that features of the algal substratum affected their abrasive or hydrodynamic properties. This variation may go some way in explaining some of the variation between the transplanted and returned colonies. Furthermore, CI colonies triggered to produce 'long' spines at HM, were found to have marginally lower spine lengths than 'long' spined colonies originating from HM. This implies that at least some genetic differentiation exists between the two sites, with respect to the

expression of spine length. Colony area indicates a degree of adaptation of the local population to the prevailing conditions. Colonies were found to be largest when situated at their natal site, transplantees being reduced in size. Higher RCP values observed for native colonies over transplantees, indicate a faster growth rate in the locals (Okamura, 1992; Bayer & Todd,1996). Finally, the higher percentage loss of experimental colonies over controls, further emphasises the degree of adaptation to the local conditions. It must however be remembered that severe stress was likely to be inflicted upon the colonies due to the initial reduction in colony size. This will consequently result in a highly exaggerated mortality rate from that of natural populations. The obvious stress acting on colonies at the HM site was wave exposure, particularly relevant to small transplantees lacking any spine protection. CI would appear to have far less hostile conditions than HM. However, the high mortality of the HM colonies transplanted to this site suggests otherwise. A possible cause of high mortality in the HM colonies is the comparatively heavy loading of silt in the water of the Menai Strait which is unfavourable to many Bryozoa (Ryland, 1976; Wood & Seed, 1980). The presence of spines in the initially transplanted colonies would encourage the deposition of silt, due to the likely increasing of the boundary layer above the colony (Riedl & Forstner, 1968). An increase in silt in the area of the colony is likely to hinder efficient lophophore feeding, resulting in a decreased feeding efficiency (Best & Thorpe, 1996).

4.4.7. Particle path measurement

The effect of 'long' spines appears to be a pronounced reduction in particle velocity over the colony surface. It has now been established that 'long' spines are produced in response to wave-related abrasion and not through water motion alone (Bayer *et al.*, 1997). Although, it appears that abrasion is the trigger, such spine assemblages may convey secondary advantages besides that of protection from direct contact. Colonies of *Membranipora villosa* are unable to feed at current velocities between 2 and 5 cm s^{-1}, measured at the colony surface (Lidgard, 1981). It was postulated that colonies were rarely exposed to such velocities and escaped the full ambient flow by living in the boundary layer. For colonies existing at least partly within the influence of the boundary layer a spatial refuge is created, protected by the highly stressful environment from predatory organisms and readily supplied with

food particles and dissolved substances. In low flow conditions, filter-feeding organisms can experience restricted particulate and dissolved substance movement to the extent that growth may be limited (Shashar *et al.*, 1996; Sebens *et al.*, 1998). The formation of encrusting colonies enables the exploitation of highly dynamic wave-swept environments. Nonetheless drawbacks arise from the formation of laminar zooidal assemblages. High shear created below lophophores in large colonies causes viscous resistance to the excurrent flow, resulting in decreased feeding efficiency (Grünbaum, 1995). However, more recently Okamura and Eckman (1997) have shown that although a degree of resistance is incurred by colonies with encrusting morphology, the overall feeding efficiency is increased in some flow conditions. Colonies of *E. pilosa* were found to function well in conditions of higher flow, compared to *Conopeum reticulum* (Okamura & Eckman,1997). Epiphytic colonies are more likely to be subjected to higher flow velocities than colonies associated with hard substrata which forms the sea bed. This is largely due to the algal fronds being held clear of the substrata, at least to a certain extent, by negatively buoyant tissue (Norton, 1991). Moving up into the water column results in an increase in flow velocity due to the diminishing influence of drag from the sea bed (Vogel, 1994). Boundary layers associated with some kelps from wave swept sites have been found to be near laminar and twice the depth predicted by boundary layer theory in unidirectional flows (Hurd *et al.*, 1997). However, fucoids occurring in shallow water are more likely to be subjected to oscillatory flows, which are not associated with stable boundary layers (Grant & Madsen, 1979; Helmuth & Sebens, 1993). Increasing the boundary layer over colonies in high flow conditions may enable lophophore extension and feeding to take place. Recently, experiments have shown clones of *Flustrellidra hispida* exposed to varying regimes of turbulent flow produce more spines in higher flow environments (Whitehead *et al.*, 1996). The lophophores of colonies with a lower number of spines were found to be adversely affected by flows of 15 cm s^{-1}. It is suggested that spines protruding from the colony surface increase the boundary layer and therefore allow efficient lophophore feeding at higher water velocities. However, Grünbaum (1997) suggests that the defensive spines produced by *Membranipora membranacea* incur high costs in the form of increased resistance to excurrent flow beneath the lophophore canopy. The numerous opesial spines of *E. pilosa* zooids might be thought to elicit similar hydrodynamic

penalties, rather than the benefits proposed herein. Such problems with increased resistance to excurrent flow may be negated by colony astogeny in *E. pilosa*. Unlike *M. membranacea*, *E.pilosa* does not form 'chimneys' allowing the escape of excurrent flows (Lidgard, 1981). This may be due to the less integrated zooidal morphology which allows easier escape of such flows. The stellate nature of many *E. pilosa* colonies, particularly those from HM, will also result in greater colony perimeter allowing excurrent flow to escape from this region, as well as more centrally behind the first polypide generation. This may well explain why the more spineous HM colonies generally exhibit a more stellate colony form. The extended median proximal spines appear to increase the boundary layer above the colony and aid rather than inhibit feeding. The orientation in settlement and growth of *E. pilosa* towards the distal part of the frond results in the newly budded zooids, which have not yet produced spines, reaping the benefits of the spines further upstream (Ryland & Stebbing, 1971). The older spineous parts of the colony are always upstream of the younger, due to the streaming of the algal thallus in the prevailing current. This may go some way to explaining the higher degree of settlement and growth orientation in exposed sites observed by Ryland & Stebbing (1971). Further investigation into the particle capture efficiency of colonies with 'long' spines compared to those with 'short' spines in high velocity flows is, however, required for the verification of the proposed advantages.

In conclusion, considerable differentiation is evident between the CI and HM populations of *E. pilosa* (see also Chapter 5). Population adaptation seems to have occurred to exploit the local conditions. This is surprising considering the extensive dispersal potential of the planktotrophic larvae of *E. pilosa*. Failure to initiate significant 'long' spine production in laboratory based experiments is highly disappointing. This is probably largely due to the failure to create sufficiently stressful conditions. Reciprocal transplants, however, revealed plasticity in spine development. It appears colony surface morphology may well have considerable influence on feeding efficiency in high flow environments.

As an aside, Marcus (1926) observed that surface spines were often associated with convex surfaces. Colonies of *M. membranacea* growing on fronds of *F. serratus* at HM, produce copious numbers of tower zooids along the convex mid

rib and edge of fronds. It seems possible that these unusual zooid types may be another example of an inducable polymorphism to prevent abrasion.

CHAPTER 5.

AN EXAMINATION OF POPULATION GENETIC STRUCTURE IN *CELLEPORELLA HYALINA* AND *ELECTRA PILOSA*, USING RAPD ANALYSIS

5.1. INTRODUCTION

5.1.1. RAPD Technique

The advent of molecular techniques to identify genetic variation within natural populations has done a great deal to revitalise present day ecological study (Weatherhead & Montgomerie, 1991; Mitton, 1994). One such technique that has become popular in some areas of research is randomly amplified polymorphic DNA (RAPD) analysis. This technique utilises synthetic decamer oligonucleotide primers of arbitrary sequence in a PCR based assay. Sections of genomic DNA are amplified through the binding of the arbitrary primers to inverted repeat sequences that are less than 3 kilobases apart. Inverted repeats may occur several times within a genome, producing a number of different lengthed products for each primer used. The products of the amplification can first be separated by electrophoresis on agarose gels and then visualized using Ultra Violet light after staining with Ethidium bromide. The RAPD procedure was first developed by Williams *et al.*, in 1990 and two other closely related techniques were established soon after. Arbitrary primed polymerase chain reaction (AP-PCR) was developed in the same year (Welsh & McCelland, 1990), followed by DNA amplification fingerprinting (DAF)(Caetano-Anolles *et al.*, 1991). These later techniques differed slightly from RAPDs in the visualisation of amplification products, PCR amplification conditions and the length of the arbitrary primers used. RAPD analysis has, however, gained the most favour.

Differences in RAPD profiles of individuals can be detected when amplification of a specific region does not occur. Absence of a fragment can be due to either a mutation at, or a large insertion between, the two annealing sites, both of which prevent amplification of the product (Williams *et al.*, 1990). However, polymorphic bands do not necessarily coincide with mutations or insertions of this type within the amplified region. It is thought that mutations can be detected outside the range of the annealing sites through the formation of secondary structures which

inhibit amplification (Bowditch *et al.*, 1993).

The RAPD technique offers a number of advantages over some other DNA markers. In contrast to DNA fingerprinting (minisatellites) (Jeffreys *et al.*, 1985) or microsatellite techniques (Tautz, 1989) RAPDs require no prior sequence knowledge. Quantities of DNA required are considerably less than those used in RFLPs (restriction fragment length polymorphisms) and no radioactive materials are required to visualise products. In addition, RAPDs, through the relatively easy application of a vast array of potential primers, allows location of polymorphisms even in highly monomorphic species, a problem often encountered with the use of allozymes (Black, 1993; Stewart & Excoffier, 1996). These advantages make RAPDs a relatively inexpensive and easy method of obtaining genetic profiles from a large number of individuals. Nevertheless, RAPDs have attracted criticism for a number of its shortcomings (reviewed in Black, 1993; Bachmann, 1994; Grosberg *et al.*, 1996). The first major drawback of the technique is the generally dominant nature of the markers obtained. The amplification products from a single locus can only be scored as either present or absent, thus preventing distinction between heterozygotes and homozygotes. Individuals with two copies of an allele can not be distinguished from those with only a single copy (Tingey & del Tufo, 1993). Some other DNA assays produce co-dominant markers, giving a higher analytical power than can be achieved using RAPDs. Co-migration of non-homologous alleles between individuals is also a problem associated with the RAPD technique (Hadrys *et al.*, 1992). However, this may be resolved either by increasing the resolution of the electrophoresis method, using acrylamide or longer agarose gels (Grosberg *et al.*, 1996), or by re-probing the PCR products via Southern hybridisation (Southern, 1975), when the co-migrating band should become labelled and thus identifiable (Smith *et al.*, 1994; Quiros *et al.*,1995). The greatest criticism of RAPDs is, however, the lack of reproducibility of profiles (Hadrys *et al.*, 1992). Causes for the lack of consistency in banding patterns range from the type of DNA extraction protocol used to obtain the template DNA (Micheli *et al.*, 1994), to the model of thermal cycler used (Penner *et al.*, 1993) and most other conceivable variables in between (reviewed in Schweder *et al.*, 1995). Adherence to an extremely stringent protocol and tests of profile repeatability are generally accepted as the remedy to the reproducibility conundrum (Clark & Lanigan, 1993; Okamura *et al.*, 1993; Grosberg, 1996). Despite all its inherent problems,

RAPD analysis remains an attractive option, due to its 'cheap and cheerful' nature.

Useful zoological applications of the RAPD technique include species identification (Bardakci & Skibinski, 1994; Coffroth & Mulawka III, 1995; Smith *et al.*, 1996), gender recognition (Lessels & Mateman, 1998), parentage analysis (Levitan & Grosberg, 1993; Jones *et al.*, 1994; Bishop *et al.*, 1996; Stott *et al.*, 1997; Tirado & Lewis, 1997) and population structuring (Okamura *et al.*, 1993; Kappe *et al.*, 1995; Dowdy & McGaughey, 1996; Grosberg *et al.*, 1996; Bielawski & Pumo, 1997; Caccone *et al.*, 1997; Todd *et al.*, 1997; Wilson *et al.*, 1997).

5.1.2. Marine invertebrate genetic population structure.

For sessile or sedentary marine invertebrates the mode of larval dispersal profoundly affects the degree of migration possible (Crisp, 1978; Cameron, 1986; Jackson, 1986). Two general modes of larval ontogeny predominate, taking the form of either a non-feeding, lecithotrophic larva present in the plankton relatively briefly (minutes to days) constrained by limited energy reserves, or a feeding, planktotrophic larva present in the plankton for longer periods (days to months) (Strathmann, 1985). The scale of dispersal generally is a consequence of time spent in the plankton and this, in turn, strongly influences geographical range and genetic structure of populations (Scheltema, 1971, 1978, 1989). Restricted dispersal capability tends to result in decreased local genetic variability, due to the effects of selection, inbreeding and drift (Slatkin, 1985). Those species releasing lecithotrophic larvae should therefore exhibit a greater degree of genetic population structuring than those with planktotrophic larvae. Conversely, extensive dispersal leads to genetic homogeneity over much larger distances, with local populations being genetically highly heterogeneous, selection and drift varying for each newly recruited cohort (Burton, 1983; Scheltema, 1989). Studies on a variety of marine organisms have demonstrated greater genetic homogeneity in species releasing planktotrophic larvae than that observed in closely related species with lecithotrophic larvae (Duffy, 1993; Hunt, 1993; Russo *et al.*, 1994; Doherty, *et al.*, 1995; Edmands & Potts, 1997; Hoskin, 1997). However, the production of a planktotrophic larva does not preclude genetic structuring at more limited scales than dispersal potential might imply, through the action of larval behaviour (Burton, 1983, 1997; Doherty, *et al.*, 1995) and/or local flow dynamics (Fevolden, 1992; Allcock *et al.*, 1997; Rogers *et al.*, 1998).

Increasingly, the phenomenon of restricted dispersal in species with pelagic larvae is thought to be commonplace in modular benthic organisms (Jackson, 1986; Jackson & Coates, 1986). However, a number of recent studies on marine molluscs with pelagic larvae have detected localised genetic structuring, but with wide scale homogenity in allele frequencies (Hurst & Skibinski, 1995; Johnson & Black, 1984; Johnson *et al.*, 1993). These characteristics have been largely attributed to highly localised selection pressure, rather than dispersal effects. The microgeographic structuring of marine species with non-pelagic larvae is increasingly being exposed through observations of population genetic structure (Duffy, 1993; Hunt, 1993; Piertney & Carvalho, 1994; Russo *et al.*, 1994; Grosberg *et al.*, 1996; Hoskin, 1997; Todd *et al.*, 1997; Miller, 1998). Such structuring may not, however, always be the case in species with lecithotrophic larvae (Borsa & Benzie, 1996).

5.1.3. Genetics of marine bryozoans

Previously, studies of the genetics of marine bryozoans have used allozyme electrophoresis to illuminate clines in the population structure of both *Schizoporella errata* along the coast of North America (Gooch & Schopf, 1971; Schopf & Gooch, 1971; Schopf, 1974) and *Alcyonidium polyoum* along the French Coast (d'Hondt & Goyffon, 1987). Allozymes have also been used to detect species differences in *Alcyonidium* spp. (Thorpe *et al.*, 1978a; 1978b). The identification of significant genetic and morphological differentiation has been established over distances of 11-13 km in *Schizoporella errata* (Schopf & Dutton, 1976). Watts (1997) detected a greater degree of population structuring in two species of bryozoans with lecithotrophic larvae (*Cryptosula pallasinana* and *Schizoporella unicornis*), than was present in two species with planktotrophic larvae (*Membranipora membranacea* and *Electra pilosa*). Differences in allele frequencies were observed over distances as small as 200 metres in *C. pallasinana.*

This study aims to provide information on the genetic structure of populations of two cheilostome bryozoans with contrasting modes of larval dispersal. Information on population structure and the amount of genetic exchange inferred from such observations, allow predictions to be made about not only population dynamics, but also how a species will respond to localized extinction due to habitat destruction. (Underwood & Fairweather, 1989; Jablonski & Raup, 1995).

5.2. MATERIALS AND METHODS

5.2.1. Study material

Colonies of both *Celleporella hyalina* and *Electra pilosa* were collected over a two day period, during September 1995. Both bryozoan species were gathered from each of two species of macro algae, *Laminaria saccharina* and *Fucus serratus*. The colonies were removed from locations while still attached to their algal substratum. Algae with associated colonies were collected at differing spatial levels. The initial spatial level took colonies from a single algal frond then from fronds 1 m, 10 m, 100 m and finally 10 Km apart. A minimum of eight separate colonies of each bryozoan species was removed from each algal frond. The majority of the samples were from Church Island, Menai Strait, North Wales (53° 13' N 4° 11' W), the exception being samples taken 10km away at Black Rocks, Beaumaris (53° 17' N 4° 6' W) also on the Menai Strait. A number of samples of *E. pilosa* from Hell's Mouth, Llyn peninsula, North Wales (52° 17' N 4° 33' W) were also taken. Additional colonies of *C. hyalina* were collected from Las Cruces, Central Chile (33° 30' S 71° 38' W) and were settled on glass plates (kindly supplied by Dr. Juan Cancino) (Fig 5.1a to 5.1c). Algal fronds were taken to the laboratory where they were washed and left in filtered sea water for 24 hrs. The colonies were then cleaned with a coarse artist's brush and examined with a Wild dissecting microscope to check for any remaining adherent organisms or parasites. Once the colonies were deemed to be clear of contamination they were carefully removed from the algal substratum using a sterile rounded scalpel blade and placed in 1.5 ml microcentrifuge tubes containing 70 % ethanol. The preserved tissue samples were kept at 4 °C until extraction of DNA could be carried out.

5.2.2. Genomic DNA extraction

The ethanol preserved bryozoan tissue was incubated at 65 °C for an hour with cell lysis solution (50 mM Tris pH8, 10 mM EDTA, 2% SDS) and 3 µl Proteinase K solution (20 mg ml^{-1}). Samples were then incubated at 55 °C for several hours with 3 µl RNAse solution (10 mg ml^{-1}). Following incubation, the samples were chilled and a protein precipitation was carried out using 5 M ammonium acetate, combined with 10 minutes centrifuging at 13000 rpm. The DNA was

precipitated from the supernatant, using isopropanol, and kept at a temperature of -20 °C for 15 minutes, after which it was centrifuged again at 13000 rpm for 20 minutes. The supernatant was then carefully decanted and the remaining DNA pellet was washed with 1 ml of 70% ethanol and vacuum dried for 10 minutes. Rehydration was achieved using 50 µl of TE buffer, shaken, and left overnight at 4 °C. All samples were run on a mini gel to test for the presence of high molecular mass DNA and to estimate concentration by comparing samples with λ DNA quantification standards (Gibco BRL).

5.2.3. Amplification conditions

The protocol used for amplification is a modified version of that used by Okamura *et al.*, (1993). Amplification reactions were conducted in 50 mM KCl, 4 mM $MgCl_2$, 10 mM Tris HCl (pH 8.3), containing 100 µM of each of dATP, dCTP, dGTP and dTTP (Pharmacia, Sweden), 0.4 µM primer (Pharmacia, Sweden), 1.0 unit of Taq DNA polymerase (Gibco BRL) and 0.25 µl of DNA suspension at a concentration of 60 ng μl^{-1}. The arbitrary decamer primers that were screened for use were those selected by Okamura *et al.* (1993). The screened primers, are listed in table 5.1. .

Table 5.1. Primer sequences screened for use with both *C. hyalina* and *E. pilosa.*

Primer No.	Okamura No.	Primer Sequence	G - C %	Used
1	15	5' CTGGCGGCTG 3'	80	
2	19	5' CATGCAGGCG 3'	70	
3	16	5' GGGTAACGCC 3'	70	
4	14	5' GGTGACGCAG 3'	70	
5	10	5' TAGCAGCGGG 3'	70	X
6	9	5' AGCAGCGTGG 3'	70	X
7	8	5' CGCAGCGTTC 3'	70	X
8	18	5' AATCGGGCTG 3'	60	X
9	17	5' GTGATCGCAG 3'	60	X
10	12	5' ATGGATCCGC 3'	60	
11	6	5' TGGTCAGTGA 3'	50	
12	2	5' TGCTCACTGA 3'	50	
13	4	5' TGGTGACTGA 3'	50	

Primer sequences with 'X' indicating primers selected for use in this study. Okamura No. indicates numbers allocated to primers in Okamura *et al.* (1993).

Selection of primers was based on their ability to produce repeatable profiles with a degree of polymorphism between individuals. The reaction mixture was overlaid with an equal volume of mineral oil and amplified in an OmniGene thermal cycler (Hybaid, UK) programmed as follows.

Table 5.2. Thermal cycler amplification conditions.

Stage	Function	Temperature °C	Time Mins.	No. Cycles
1	Denature	94	2.00	1
2	Denature	94	0.10	
	Annealing	40	0.30	40
	Extension	72	1.30	
3	Final Ext.	72	5.00	1
	Room Temp	28	0.10	1

Controls were run with either no template or substratum (seaweed) template. Each individual was re-amplified with each primer to ensure reproducibility of banding profiles. The amplification products and 100 bp ladder (Gibco, BRL) were electrophoresed at 120 Volts for 3 hours on 1.2 % agarose gels with 1X TBE buffer (0.089m Tris borate, 0.002m EDTA) and stained with Ethidium bromide. Gels were visualised using UV illumination and photographed with 667 Polaroid film.

5.2.4. Statistical analysis

For each primer Polaroid photographs of gels were scored for the presence or absence of bands of a specific size. Bands were scored in a highly conservative manner to eliminate false results from weakly amplified fragments. A genetic distance matrix, based on band sharing was calculated with unweighted pair-group mean arithmetic average (UPGMA) cluster analysis (Sneath & Sokal, 1973) and results displayed as a dendrogram using the STATISTICA package (StatSoft, Inc.1995). The raw binary data (Appendix 2.1) were converted into a matrix of Euclidean squared distances using AMOVA-PREP (M. P. Miller, Northern Arizona University) then subjected to an AMOVA (Analysis of Molecular Variance) (Excoffier *et al.*, 1992) using the ARLEQUIN program (Version 1.1; S. Schneider *et al.*, Geneva). AMOVA calculates an F_{st} analogue ϕ_{st} (Wright, 1951; Weir & Cockerham, 1973) that allows variation both within and between populations to be estimated, assuming

populations are in Hardy-Weinberg equilibrium. Finally, genetic distance matrices created from Slatkin's linearised Fst's and UPGMA cluster analysis data, were used to perform matrix correspondence tests against geographic distance (Sokal, *et al.*, 1997) (Appendix 2.2).

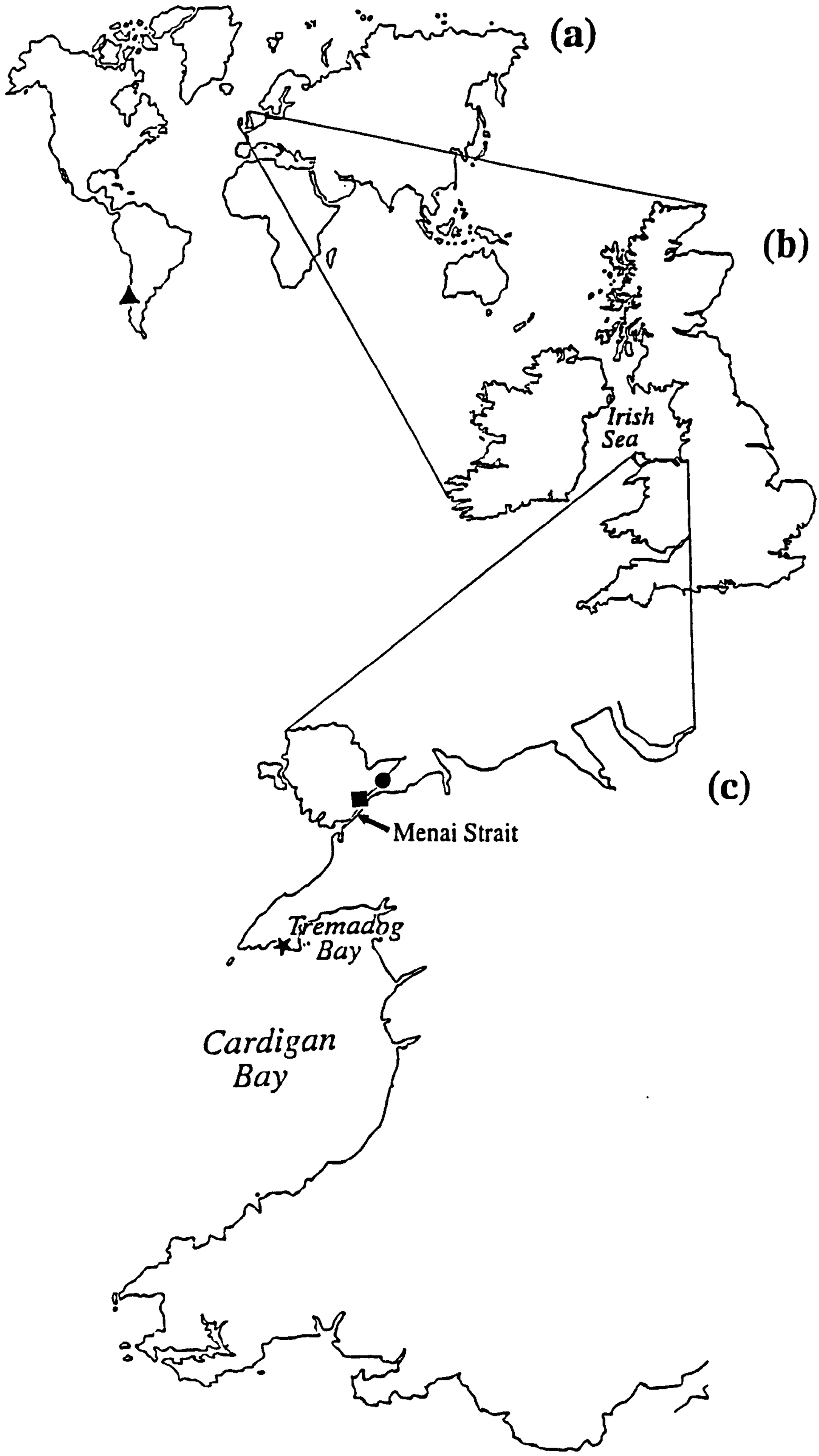


Figure 5.1. Site map showing sampling locations in Wales , U.K. and Chile. (a) World, (b) United Kingdom, (c) Wales. ■=Church Island, ●=Beaumaris, ★=Hell's Mouth, ▲=Las Cruces, Chile.

5.3. RESULTS

RAPD profiles recorded on Polaroid photographs yielded 104 bands for *C. hyalina* and 53 bands for *E. pilosa* (Table 5.3 Fig. 5.2 & 5.3).

Table 5.3. Number of monomorphic and polymorphic bands scored from RAPD profiles, for both *C. hyalina* and *E. pilosa* over the range of primers used.

	Primer 5		*Primer 6*		*Primer 7*		*Primer 8*		*Primer 9*	
	M	P	M	P	M	P	M	P	M	P
C. hyalina	2	16	2	16	1	20	2	25	3	17
E. pilosa	2	10	1	10	1	11	3	7	2	6

Data presented are, M =number of monomorphic bands, P =number of polymorphic bands.Chilean sample excluded, due to absence of bands in common with Welsh samples.

UPGMA cluster analyses are displayed as dendrograms of Euclidean distances for both *C. hyalina* and *E. pilosa* (Figs. 5.4 & 5.5). Multi-dimensional scaling scatterplots are also presented for both species (Figs. 5.6 to 5.9). Such Euclidean distance representations do not give a strict measure of genetic distance, but rather provide a distance based on RAPD phenotypes. Dendrograms of *E. pilosa* show little structure with distance or substratum type, with the exception of the Hell's Mouth samples which appear to be distinct from the Menai Strait samples. Scatterplots reinforce this observation and highlight the randomness of samples from the Menai Strait, particularly when the Hell's Mouth population is excluded (Fig. 5.9). The *C. hyalina* dendrograms indicate considerably more population structuring than those of *E. pilosa*. The Chilean samples are clearly distinct from the Welsh samples, which might be expected, as no bands are shared by individuals from the two sites (Figs.5.4 & 5.6). Also it is evident that considerable differences are apparent between the Church Island and Beaumaris sites, and to a lesser extent between the samples within Church Island (Figs. 5.4 & 5.7).

AMOVA results are displayed in Tables 5.4 to 5.7. No significant affect of substratum was detected for either *C. hyalina* or *E. pilosa*. Both species show significant variation among populations within substrata, indicating that structuring occurs in both species. However, *C. hyalina*, shows a slightly higher percentage variance (5.29 %), than that of *E. pilosa* (1.61 %), implying a somewhat greater

degree of structuring in this species. If the Hell's Mouth samples are excluded from the analysis of *E. pilosa* populations, the significant within substrata variation among populations is no longer present (Table 5.7). This suggests that there is no structuring within the Menai Strait population of *E. pilosa*. The majority of the variance is found within populations indicating a high degree of genetic heterogeneity for both bryozoan species.

Table 5.5. AMOVA for 80 individuals of *C. hyalina*, from two algal substrates, *Fucus serratus* and *Laminaria saccharina*.

Each population consists of 8 individuals. Chilean samples excluded.

Source of variation	*d.f.*	*SS*	*Variance component*	*Percentage variance*	*P-value**
Among substrata	1	51.98	0.00	0.00	0.49129
Among populations (within substrata)	8	429.30	2.06	5.29	<0.0005
Within populations	70	2597.87	37.11	94.82	<0.0005

* Significance test (10,100 permutations)

Table 5.6. AMOVA for 80 individuals of *E. pilosa*, from two algal substrates, *Fucus serratus* and *Laminaria saccharina*.

Each population consists of 8 individuals.

Source of variation	*d.f.*	*SS*	*Variance component*	*Percentage variance*	*P-value**
Among substrata	1	20.81	0.00	0.00	0.75723
Among populations within substrata	8	178.42	0.32	1.61	<0.0005
Within populations	70	1380.75	19.72	98.58	<0.0005

* Significance test (10,100 permutations)

Table 5.7. AMOVA for 72 individuals of *E. pilosa*, from two algal substrates, *Fucus serratus* and *Laminaria saccharina*.

Each population consists of 8 individuals. Hell's Mouth samples are excluded.

Source of variation	*d.f.*	*SS*	*Variance component*	*Percentage variance*	*P-value**
Among substrata	1	20.29	0.02	0.14	0.15792
Among populations (within substrata)	7	135.38	0.00	0.00	0.63911
Within populations	63	1230.62	19.53	99.99	0.54980

Significance test (10,100 permutations)

Physical and genetic distance are found to be significantly associated when using matrix correspondence tests and comparing both Slatkin's linearised Fst's and UPGMA cluster analysis against geographical distance matrices for *C. hyalina* populations (Table 5.8). In contrast, genetic distance was found not to correlate significantly with physical distance for *E. pilosa* over the same area (Table 5.9). However, if the Hell's Mouth samples are included in the analysis, a significant correlation with geographical distance is observed using both UPGMA (correlation coefficent = 0.9852, p = <0.05) and Slatkin's linearized Fst (correlation coefficent = 0.9834, p = <0.05) matrices.

Table 5.8. Matrix correspondence tests for physical distance against genetic distance. Genetic distance obtained using Slatkin's Linearised Fst and Unweighted pair-group mean (UPGMA), for *C. hyalina* on separate algal substrates. Outlying Chilean population is excluded.

	Slatkin's Linearised Fst		*UPGMA*	
	C	P	C	P
Fucus serratus	0.8164	<0.05*	0.7165	<0.05*
Laminaria saccharina	0.9507	<0.05*	0.9421	<0.05*

Data presented are Correlation coefficients (C), with P-values (P). *, significant values. Probabilities are derived from 10,000 permutations.

Table 5.9. Matrix correspondence tests for physical distance against genetic distance. Genetic distance obtained using Slatkin's linearised Fst and Unweighted pair-group mean (UPGMA), for *E. pilosa* on separate algal substrates. Outlying Hell's Mouth population is excluded.

	Slatkin's Linearised Fst		*UPGMA*	
	C	P	C	P
Fucus serratus	31.67	0.4922	29.02	0.5002
Laminaria saccharina	25.34	0.5531	24.96	0.7268

Data presented are Correlation coefficients (C), with P-values (P). *, significant values. Probabilities are derived from 10,000 permutations.

Matrix correspondence tests were conducted on data separated by substrata prior to the AMOVA analysis (Tables 5.5 to5.7). AMOVA analysis indicated no significant difference between the colonies found on either substrata. Matrix correspondence tests produced very similar results for both *L. saccharina* and *F. serratus* indicating that grouping the data would have little effect on the overall results or conclusions.

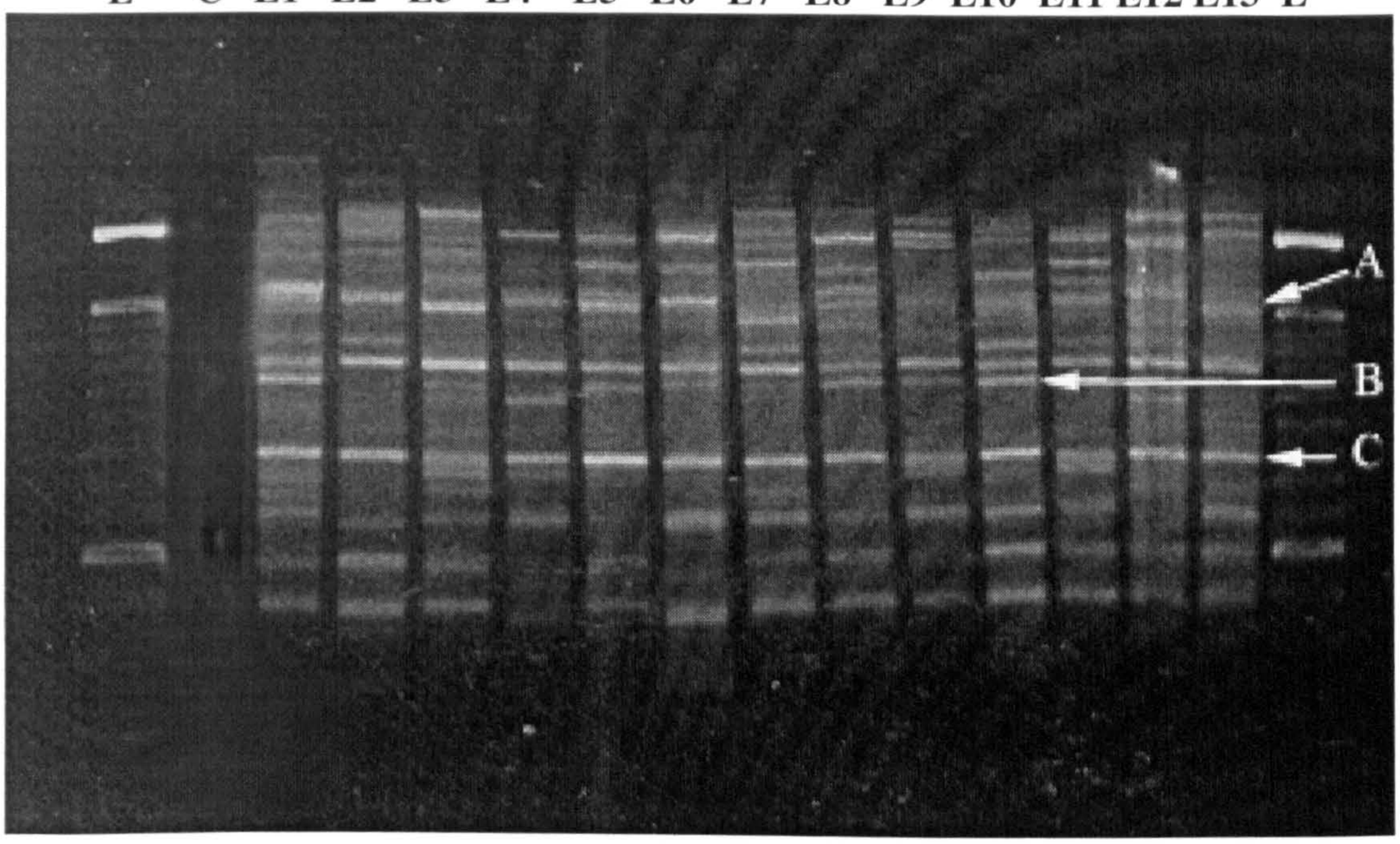


Figure 5.2. Polaroid photograph of RAPD profiles obtained using primer 5, for samples of *E. pilosa* from Hell's Mouth. L = 100 bp ladder, C = Control, E1-E13 = samples, A= Unscored band, B = Polymorphic band, C = Monomorphic band.

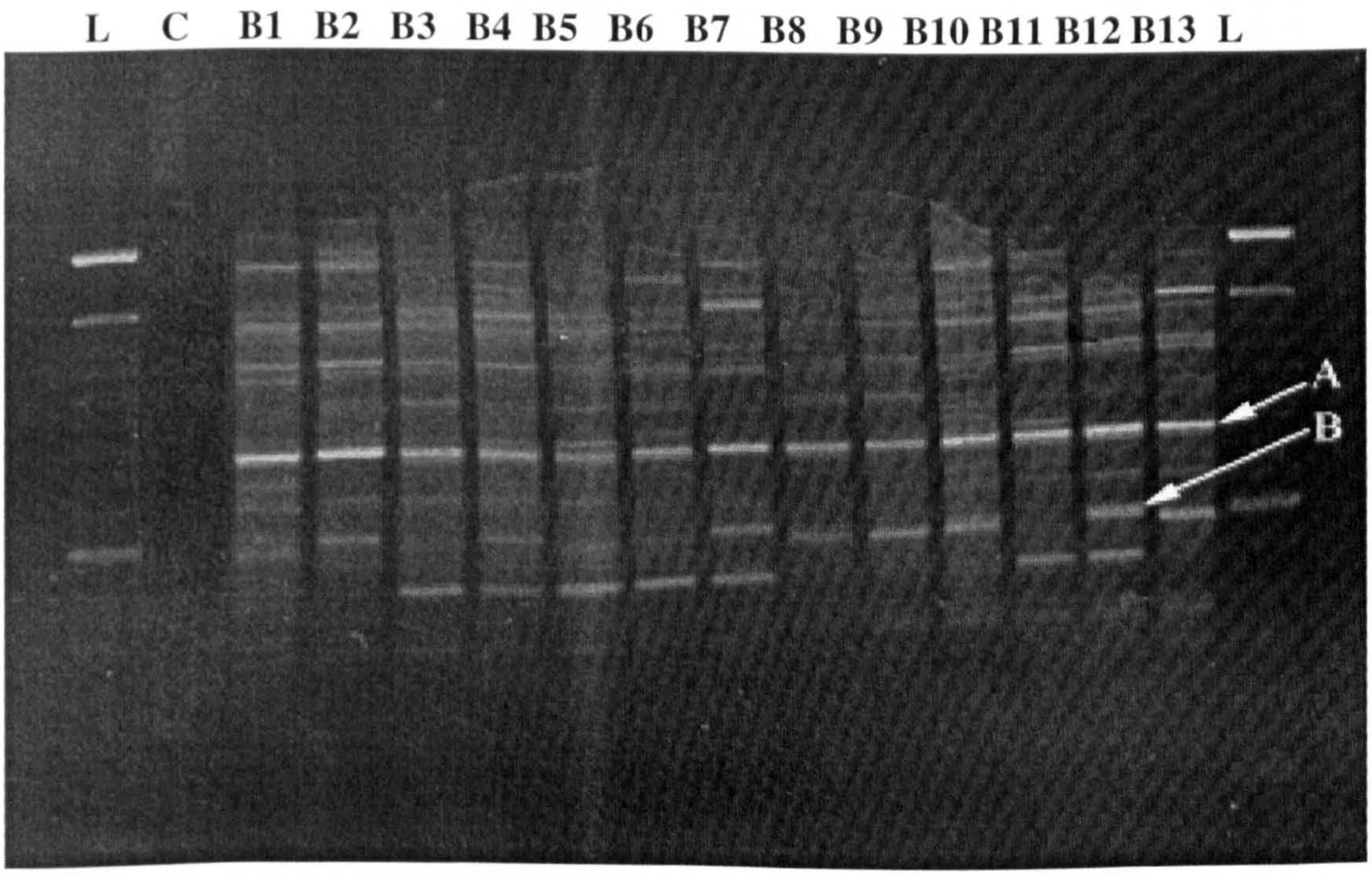


Figure 5.3. Polaroid photograph of RAPD profiles obtained using primer 7, for samples of *C. hyalina* from Church Island. L = 100 bp ladder, C = Control, B1-B13 = Samples, A= Monomorphic band, B= Polymorphic band.

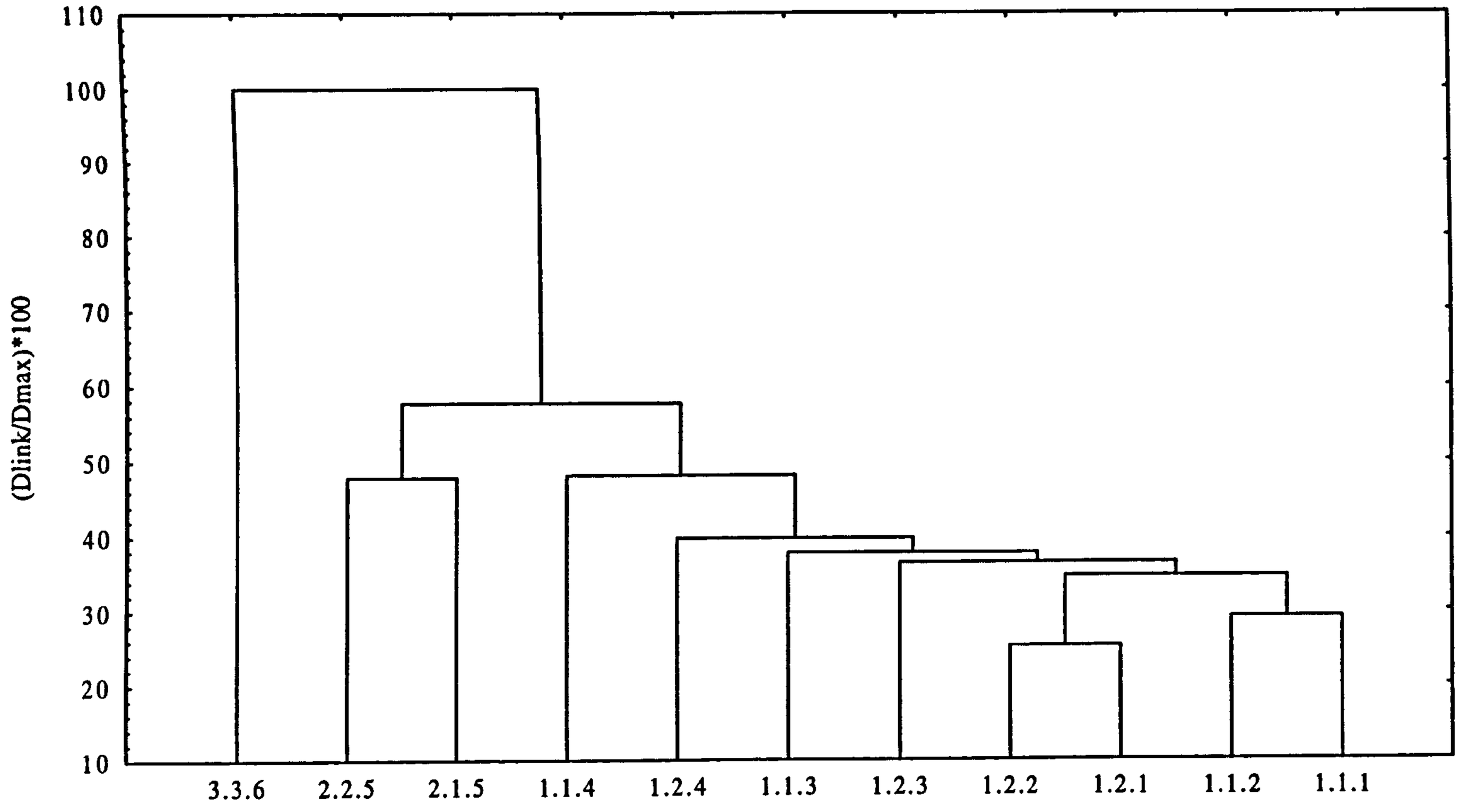


Figure 5.4. UPGMA dendrogram of Euclidean distances from RAPD data for 88 individuals of *C. hyalina*. Each sample constitutes 8 individuals.

The first digit on X axis = Site (1=Church Island, 2=Beaumaris, 3=Chile), second digit = Algal substrate (1=*Fucus serratus*, 2=*Laminaria saccharina*, 3=Glass), third digit = Distance relative to sample 1.1.1 (1=0 m, 2=1 m, 3=10 m, 4=100 m, 5=10 km, 6=13000 km). Therefore, sample 1.2.4 is from Church Island found on *L. saccharina*, at a distance of 100 m from sample 1.1.1. Y axis scale, (Dlink/Dmax*100) = Distance expressed in terms of the largest observed difference.

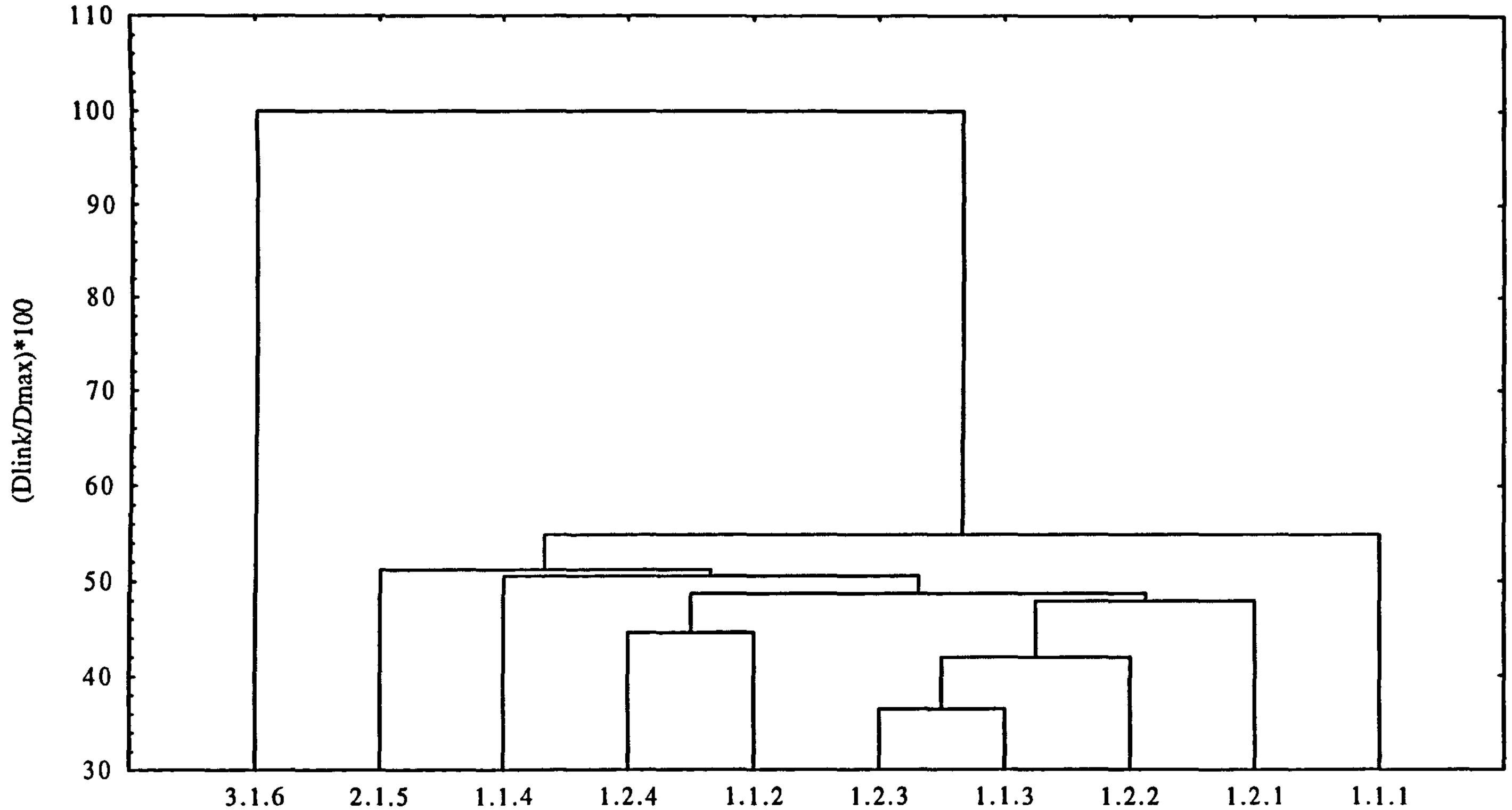


Figure 5.5. UPGMA dendrogram of Euclidean distances from RAPD data for 80 individuals of *E. pilosa*. Each sample constitutes 8 individuals. The first digit on X axis = Site (1=Church Island, 2=Beaumaris, 3=Hell's Mouth), second digit = Algal substrate (1=*Fucus serratus*, 2=*Laminaria saccharina*), third digit = Distance relative to sample 1.1.1 (1=0 m, 2=1 m, 3=10 m, 4=100 m, 5=10 km, 6=70 km). Therefore, sample 1.2.4 is from Church Island found on *L. saccharina*, at a distance of 100 m from sample 1.1.1. Y axis scale, (Dlink/Dmax*100) = Distance expressed in terms of the largest observed difference.

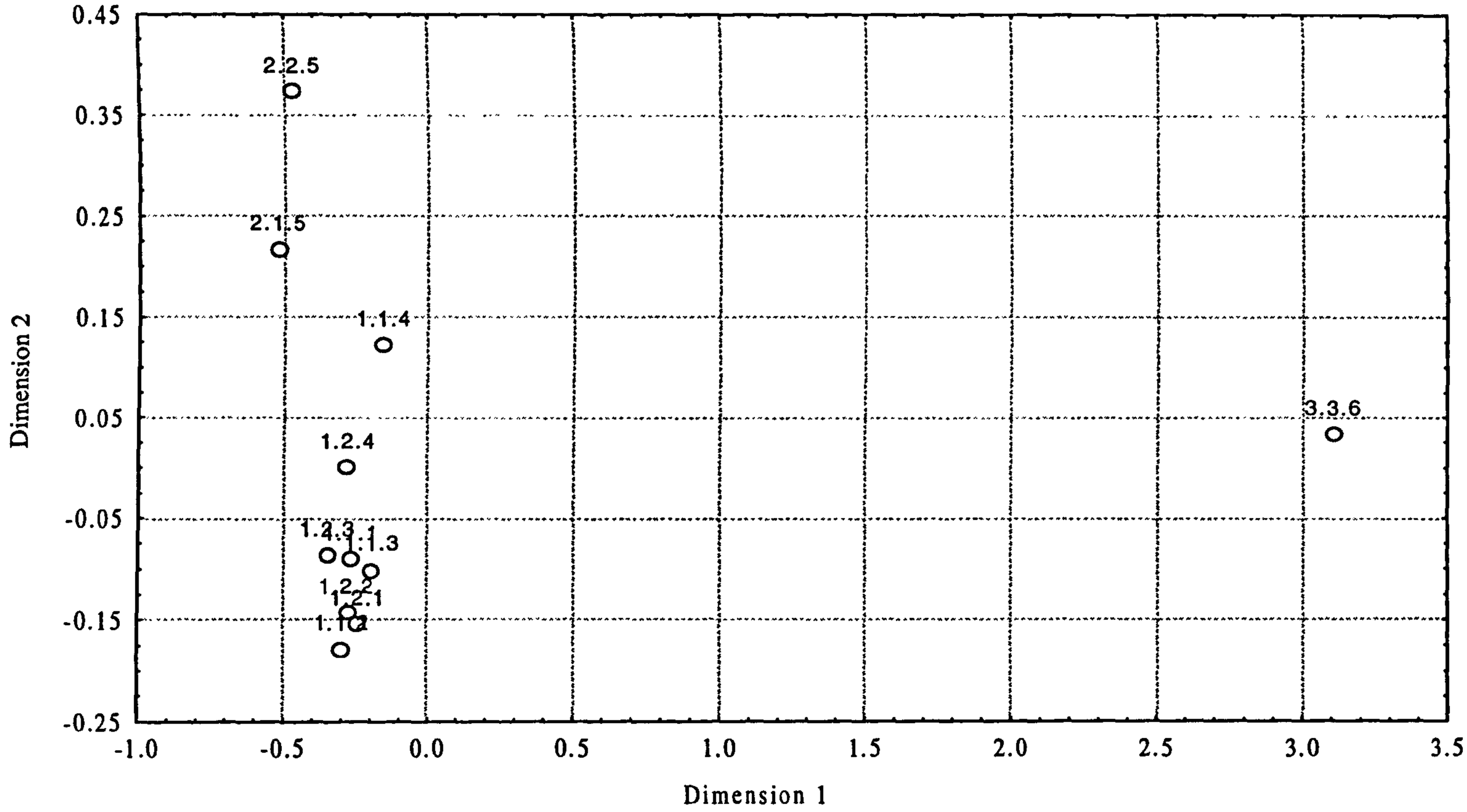


Figure 5.6. Multi-dimensional scaling 2D scatterplot from RAPD data for 88 individuals of *C. hyalina*. Each sample constitutes 8 individuals. The first digit on sample code = Site (1=Church Island, 2=Beaumaris, 3=Chile), second digit = Algal substrate (1=*Fucus serratus*, 2=*Laminaria saccharina*, 3=Glass), third digit = Distance relative to sample 1.1.1 (1=0 m, 2=1 m, 3=10 m, 4=100 m, 5=10 km, 6=13000 km). Therefore, sample 1.2.4 is from Church Island found on *L. saccharina*, at a distance of 100 m from sample 1.1.1.

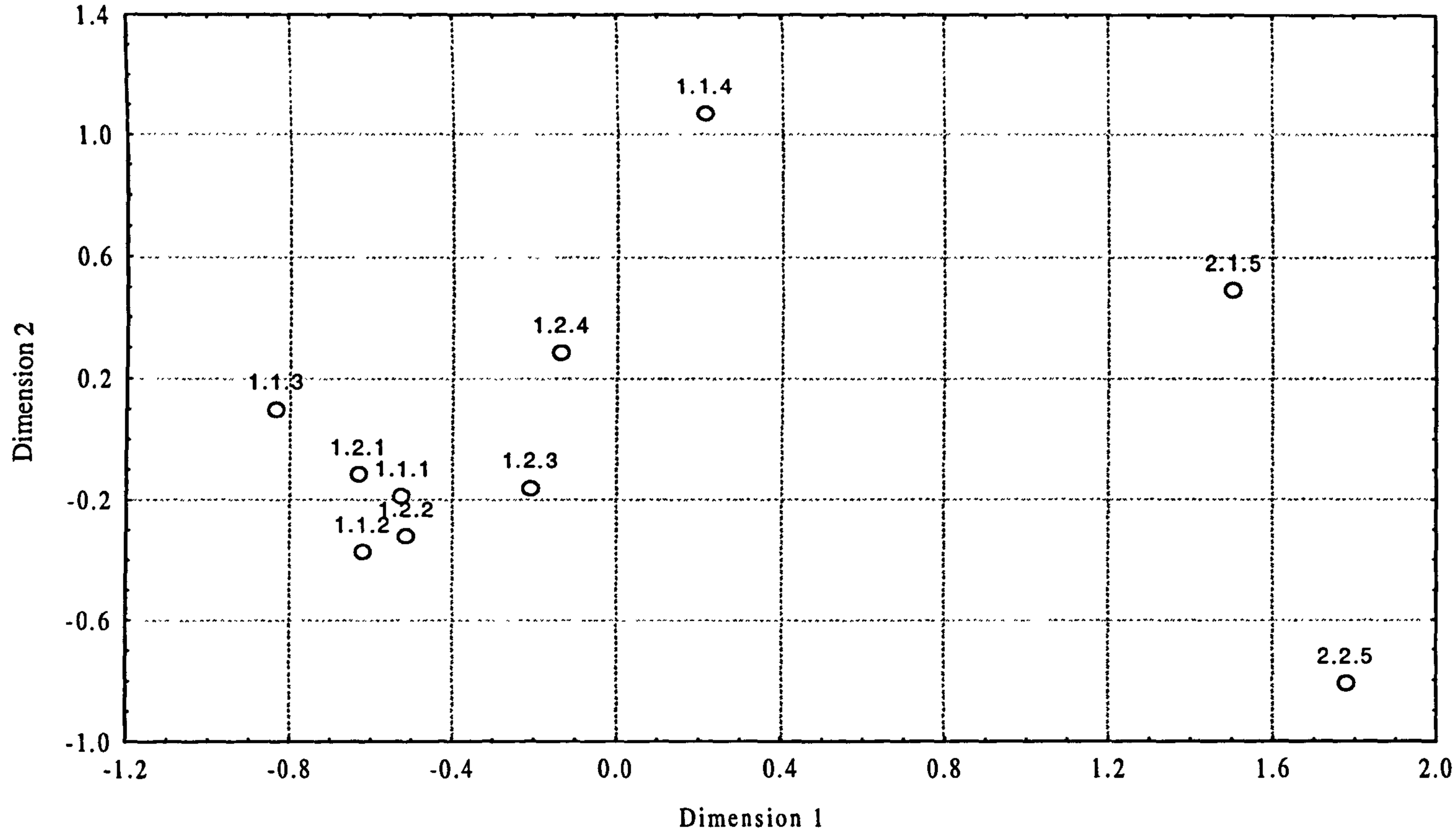


Figure 5.7. Multi-dimensional scaling 2D scatterplot from RAPD data for 80 individuals of *C. hyalina*, excluding the Chilean samples. Each sample constitutes 8 individuals.

The first digit on X axis = Site (1=Church Island, 2=Beaumaris), second digit = Algal substrate (1=*Fucus serratus*, 2=*Laminaria saccharina*), third digit = Distance relative to sample 1.1.1 (1=0 m, 2=1 m, 3=10 m, 4=100 m, 5=10 km). Therefore, sample 1.2.4 is from Church Island found on *L. saccharina*, at a distance of 100 m from sample 1.1.1.

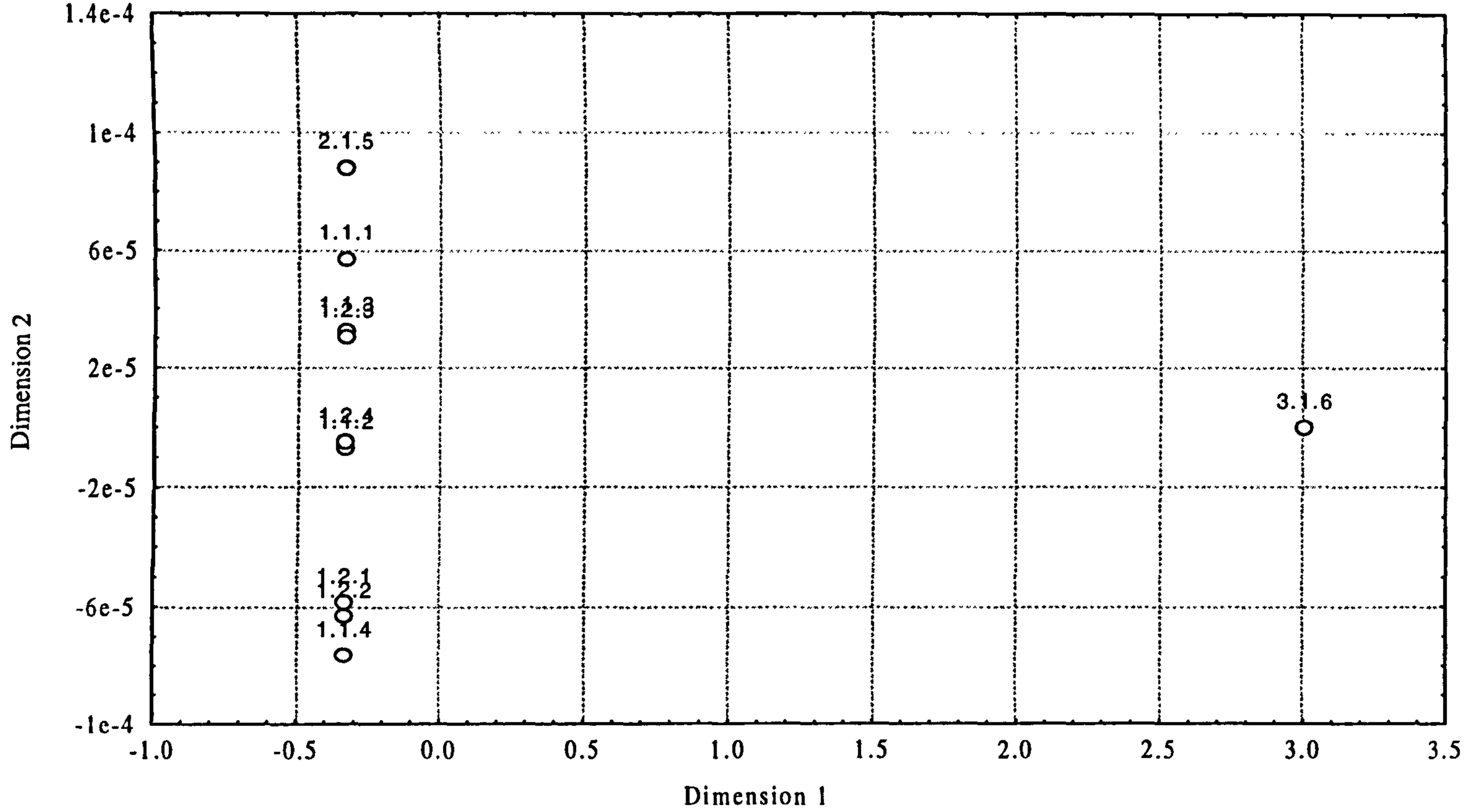


Figure 5.8. Multi-dimensional scaling 2D scatterplot from RAPD data for 80 individuals of *E. pilosa*. Each sample constitutes 8 individuals. The first digit on sample code = Site (1=Church Island, 2=Beaumaris, 3=Hell's Mouth), second digit = Algal substrate (1=*Fucus serratus*, 2=*Laminaria saccharina*), third digit = Distance relative to sample 1.1.1 (1=0 m, 2=1 m, 3=10 m, 4=100 m, 5=10 km, 6=70 km). Therefore, sample 1.2.4 is from Church Island found on *L. saccharina*, at a distance of 100 m from sample 1.1.1.

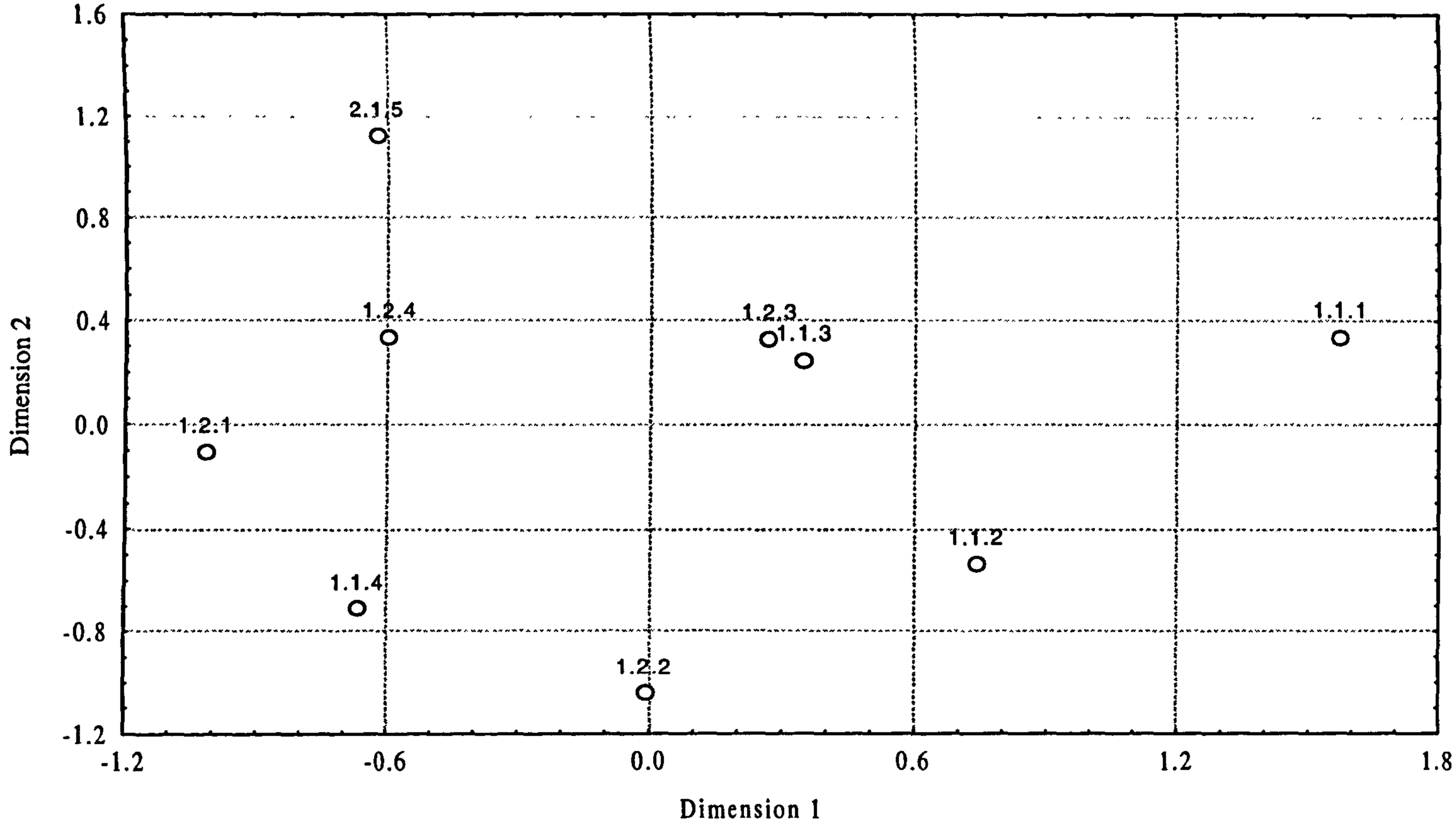


Figure 5.9. Multi-dimensional scaling 2D scatterplot from RAPD data for 72 individuals of *E pilosa*, excluding the Hell's Mouth samples. Each sample constitutes 8 individuals.

The first digit on X axis = Site (1=Church Island, 2=Beaumaris, 3=Hell's Mouth), second digit = Algal substrate (1=*Fucus serratus*, 2=*Laminaria saccharina*), third digit = Distance relative to sample 1.1.1 (1=0 m, 2=1 m, 3=10 m, 4=100 m, 5=10 km). Therefore, sample 1.2.4 is from Church Island found on *L. saccharina*, at a distance of 100 m from sample 1.1.1.

5.4. DISCUSSION

The mode of larval dispersal is reflected in the contrasting genetic population structure of the two bryozoan species examined. Results for *C. hyalina* indicate that a relationship exists between genetic and geographic distance, which leads to localised population structure. Although, it is to be expected that the more restrictive mode of dispersal characteristic of lecithotrophic larvae should create population structuring at smaller spatial scales than that of planktotrophic larvae, given a moderate tidal current and the relatively exposed nature of an algal frond in the water column, dispersal of *C. hyalina* larvae is likely to occur over several hundred metres. Fronds of laminarian and fucoid algae are held clear of the substratum, to at least a certain extent, by rigid stipes and/or negatively buoyant tissues (Norton, 1991). Laboratory experiments have established that settlement of *C. hyalina* larvae normally occurs within 4 hours (Ryland, 1960; Cancino & Hughes, 1988) and if settlement does not occur within this period the larva is unable to metamorphose (Orellana *et al.*, 1996). Extending the period of larval swimming has also been found to decrease early colony size. In Chilean *C. hyalina* (Orellana & Cancino, 1991) and *Bugula stolonifera* (Woollacott *et al.*, 1989), a reduction in initial size has been found to affect eventual reproductive output and increase the likelihood of fatal overgrowth (Ryland, 1981; Cancino & Hughes, 1987; Stocker, 1991). It must therefore be assumed most larvae will settle very soon after release, so avoiding energetic costs that would inhibit their subsequent survival and reproductive success. Nevertheless, the potential for dispersal of a larval propagule in the Menai Strait might be expected to be considerable, given a period for settlement of 4 hours and tidal current speeds of up to 1.2 m sec^{-1} (Harvey, 1968).

A computer model of particulate transport in the Menai Strait (Sherwin *et al.*, 1997) was used to predict the maximum possible distance travelled by *C. hyalina* larvae. Larval release was simulated from both Church Island and Beaumaris, under neap and spring tide conditions. Larvae were found not to be exchanged between the two sites, even during spring tides, when transport is at its maximum (T. J. Sherwin, pers. comm.). It seems likely that no direct gene flow occurs between the Church Island and Beaumaris *C. hyalina* populations. *C. hyalina* does occur, however, as

isolated colonies on patches of *F. serratus* along the Strait (Appendix 2.3), which may act as stepping stones between the two sites.

The period which larvae are present in the plankton is generally considered to relate directly to the amount of dispersal possible by sessile marine invertebrates (Crisp, 1978). The role of gametes as agents of dispersal in the marine environment is less well documented. Gametes which remain viable for long periods offer the potential to increase gene flow between populations, excluding the confounding influence of gametic incompatibility (Grosberg, 1991). Nevertheless, the likelihood of fertilisation success decreases dramatically with increased distance through dilution effects (Pennington, 1985; Denny & Shibata, 1989; Levitan, 1991; Brazeau & Lasker, 1992) and sperm competition (Yund & McCartney, 1994). Evidence of self-fertilisation in Welsh *C. hyalina* in the absence of conspecific sperm, has been presented previously (Hunter & Hughes, 1993a), albeit with a marked reduction in larval viability. More recent studies have yielded little indication of selfing, suggesting obligatory cross-fertilisation in most genotypes (P. Manríquez & R. N. Hughes, pers. comm.; but see Chapter 6). It is generally accepted that most Bryozoa possess the capacity to cross fertilise (Ryland, 1976). Recently, 2 hours has been established as the maximum time period over which sperm remain viable in the water column (P. Manríquez & R. N. Hughes, pers. comm.). In light of this finding, sperm can be considered to have the potential to travel half the distance of larvae, excluding effects of differing degrees of motility and behaviour. Data presented here imply that gene flow is sufficiently limited, through restricted dispersal of larvae and/or gametes, to allow genetic differentiation to arise over distances of metres. This is even more surprising considering the likelihood of some movement of colonies via rafting on detached seaweed. Rafting is the movement of organisms which are attached to mobile floating substratum and is increasingly being recognised as a means by which sessile or sedentary organisms can achieve long distance dispersal (Highsmith, 1985; Helmuth *et al.*, 1994; Ingólfsson, 1995). The regular sloughing of fragments of algal frond, typical of *L. saccharina* (Parke, 1948), may provide ample opportunity to move further than is possible by alternative means. Periods of highest colony density occur, however, when frond loss is at its lowest in late Summer and early Autumn (Cancino, 1986), thus reducing the probability of transport of viable colonies by this means.

The present results indicate that no significant genetic variation was found to occur between populations occurring on the two algal substrates examined (Table 5.5). Larvae of *C. hyalina* have been shown to have no overriding preference for either *F. serratus* or *L. saccharina* when settling, unlike some other common epiphytic bryozoans (Ryland, 1960). In the absence of algal specific larval settling behaviour, local oceanographic factors appear to be the dominant influence on the movement of larvae. The existence of population structuring on a relatively small scale seems to be a result of limited dispersal, well below the potential maximum for lecithotrophic larvae. Indeed, in environments where fine-scale heterogeneity is commonplace there should be no selective advantage in long distance dispersal (Jackson & Coates, 1986).

The planktotrophic larvae of *E. pilosa* should disperse over large distances resulting in a population with limited genetic structuring over a relatively wide geographical scale. The reduction in the percentage variance for *E. pilosa* obtained from the AMOVA compared to that of *C. hyalina* indicates a diminished level of structure in this species (Tables 5.5 to 5.7). No obvious structuring of populations is evident from the dendrograms or scatterplots, other than the considerable difference exhibited by the Hell's Mouth population. These observations are reinforced by the results from the matrix correspondence tests, which show no significant relationship between genetic and geographic distance in the population of *E. pilosa* from the Menai Strait. In contrast, the Hell's Mouth *E. pilosa* population did appear to display large genetic differences from that of the Menai Strait populations, where a geographical separation of some 70 km exists between the two areas. Variations in allele frequencies have been observed between Plymouth and the Irish Sea populations of *E. pilosa*, prompting speculation on a generally northward movement of larvae (Watts, 1997). Differentiation in allozymes between the Plymouth and Welsh populations of *E. pilosa*, have also been detected by d'Hondt and Goyffon (1993).These investigators also found that *E. pilosa* samples from two populations in Anglesey and South Wales differed for two enzyme systems, seemingly corroborating a unidirectional larval migration hypothesis. The present study also indicates considerable genetic differences in an *E. pilosa* population only 70 km south of the main study area (Church Island). The length of the pelagic phase of cyphonautes larvae is not well documented, although the larvae of *Membranipora*

membranacea, another cheilostome, have been kept alive for 8 weeks (Yoshioka, 1982a). Colonies possessing eggs and sperm are mainly found in late summer (Hughes 1986; Ryland & Hayward, 1977). Newly settled ancestrulae are most common between August and October, but they are present throughout the year (Ryland & Hayward, 1977; Wood, 1983). Information on the general direction of marine currents in the Irish Sea indicate a weak northerly flow (Rawe, 1983; A. E. Hill, pers. comm.). However, surface currents are strongly dependent on the wind direction which can change and rapidly reverse the direction of flow (Davies & Jones, 1992). Nevertheless, the prevailing wind direction over the main period which *E. pilosa* larvae are present in the plankton is from the South West (Lumby, 1950 to 1958), suggesting a generally Northward movement of larvae. This presents the possibility of the existence of a cline in the *E. pilosa* population running northward along the coast of Wales. Another possible oceanographic cause of such a large differentiation between Church Island and Hell's Mouth populations of *E. pilosa*, is the presence of a tidal front created by stratification in the Tremadog Bay area, which impinges on Hell's mouth during the summer months (Beggs, 1974; Rawe, 1983). The formation of such a tidal front would lead to a southward water movement into Cardigan Bay, greatly limiting gene flow north towards Anglesey (Rawe, 1983; A. E. Hill, pers. comm.). This situation would prevail until water stratification was broken down by mixing of the water column due to storm conditions (Rawe, 1983). Reproduction in *E. pilosa* is generally assumed to be through cross-fertilisation (Silén, 1966; Hughes, 1986). Male gametes are released and eventually captured by a conspecific's lophophore, thus triggering the liberation of the ova which is thought to be fertilised immediately outside the intertentacular organ (Silén, 1966). The duration of sperm viability in *E. pilosa* is uncertain. The degree of gene flow through the movement of male gametes is therefore unknown. Nonetheless, it seems probable that successful fertilisations are restricted to a very localised area, due to the effects of dilution (Pennington, 1985). No genetic structuring associated with the different algal substrates was identified. This is not unexpected, due to the highly catholic nature of substratum *E. pilosa* is found to settle upon (Ryland & Hayward, 1977).

Despite its well documented drawbacks, RAPDs appears to be a technique that is extremely adept at detecting polymorphisms among individuals, often more so than in other techniques such as allozymes (Aagaard *et al.*, 1995; Todd *et al.*, 1997;

Sydes & Peakall, 1998). Recently, microsatellite markers have been isolated for *C. hyalina* (Hoare *et al.*, 1998). Using samples from this study, insufficient resolution was achieved to distinguish between populations from Church Island and Beaumaris (K. Hoare. pers. comm.). However, the inability to detect differences at this geographical scale may only reflect the unsuitability of these particular microsatellite markers. In the present study, the RAPD technique has proved to be a highly successful way to obtain insight into the *in situ* genetic structure of two bryozoan species with contrasting methods of larval dispersal.

CHAPTER 6.

AN ASSESSMENT OF THE LIKELIHOOD OF SPECIATION BETWEEN CHILEAN AND WELSH *CELLEPORELLA HYALINA*

6.1. INTRODUCTION

6.1.1. Speciation

Species are groups of organisms, between which genetic exchange is prevented by reproductive isolating mechanisms. This statement represents the basic premise of the most widely accepted definition of species, the biological species concept (BSC) (Dobzhansky, 1937; Mayr 1942; 1963). Numerous alternative species concepts have since been proposed (Cracraft, 1983; Paterson, 1985; Templeton, 1989) but have not been as well received (Coyne, 1994; reviewed in Gosling, 1994; Avise, 1994). An attempt to incorporate the strongest points of the BSC and more recent phylogenetic concepts by Avise & Ball (1990) resulted in the conception of the concordance principle. This more recent theory still retains reproductive isolation as the main parameter enabling the assignment of species status. The mechanisms of reproductive isolation can operate either before fertilisation, prezygotic mechanisms, or after fertilisation, postzygotic mechanisms (Coyne, 1992; Coyne & Orr, 1998). Prezygotic isolation may take the form of mate choice or gametic incompatibility, whereas postzygotic isolating mechanisms include sterility, hybrid inviability and hybrid breakdown. A number of models have been suggested where genes for such isolating mechanisms can become fixed in natural populations. The first of these models is allopatric speciation (Mayr, 1942) where a barrier to gene flow isolates two sections of an extensive ancestral population. Once isolated, genetic divergence occurs through random drift, changes in selection pressures or the accumulation of incompatible mutations. Mayr (1963) later suggested peripatric speciation, which involves the isolation of only a small fraction of a larger population, rather than the splitting of two large areas, as is the case for allopatric speciation. However, there is some doubt whether a clear distinction can be made between peripatric and allopatric models of speciation (Barton & Charlesworth, 1984). Two other models on the theme

of peripatric speciation are worthy of note, founder-flush speciation (Carson, 1975) and genetic transilience (Templeton, 1980). These two models fundamentally involve rapid population expansion after a rare colonisation event. In such circumstances random genetic drift is reduced and advantageous alleles rapidly become established. The parapatric model of speciation, on the other hand, occurs where localised selection pressure causes divergence of a population, with the formation of a cline and eventual isolation at either end (Endler, 1977). Finally, the sympatric speciation model applies where diversifying natural selection allows the coexistence of two morphs, which diverge sufficiently within the same area to facilitate the formation of reproductive isolation (Maynard Smith, 1966). However, there is scant evidence of the natural occurrence of sympatric speciation. Doubts have also been raised over the probability of genes coding for the two morphs being tightly linked to genes for mating preference (Futuyma & Mayer, 1980; Felsenstein, 1981).

In the past, the majority of research into speciation has been restricted to the terrestrial realm. However, the causes of speciation in marine systems are increasingly being sought with the help of an expanding arsenal of molecular techniques (Palumbi, 1992; Skibinski, 1994). It is generally thought that marine organisms with relatively low dispersal often exhibit localised physiological adaptation and have high rates of speciation (Jablonski, 1986). In contrast, marine species with highly dispersed planktonic larvae require thousands of kilometres to achieve geographic isolation (Palumbi, 1992). However, growing evidence suggests that there is a greater subdivision of marine species, with high dispersal potential, than was previously thought (Hilbish, 1996).

6.1.2. Phylogeography

Mitochondrial DNA (mtDNA) provides a relatively amenable way to examine phylogeny and population structure. This is due largely to its relative ease of isolation from the genome, rapid rate of evolution and maternal mode of inheritance (Brown, 1983). Animal mtDNA is haploid, non-recombining and, unlike the nuclear genome, contains very few duplicate or non-coding regions (Hayashi, 1985; Gray, 1989). The rapid evolution of mtDNA allows intraspecific population structure to be examined. MtDNA clades (phylogenetic lineages) have been shown to be geographically localised. Phylogeography is the term now used to describe the study of the processes

giving rise to genealogical distributions (Avise *et al.*, 1987). Rate of evolution of the mitochondrial genome is in the region of 5-10 times faster than that of single copy nuclear DNA (Brown *et al.*, 1982). This is largely thought to be the result of a high frequency of point and length mutations (Brown *et al.*, 1982). Three possible types of sequence changes exist, rearrangements, indels (additions or deletions) and substitutions. Of these three possibilities substitutions are the most common, taking the form of either transitions (purine to purine and pyrimidine to pyrimidine) or transversions (purine to pyrimidine and vice versa). Transitions are generally more frequent than transversions, to the extent that the most variable third codon position can become saturated with transitions (Brown, 1983). Consequently, the third position may not be a reliable indicator of evolutionary relationship beyond thirty to forty percent sequence divergence. For this reason transitions and transversions are often weighted differently in phylogenetic analysis. The development of 'universal primers' has increased the use of mtDNA. Universal primers are designed to anneal to highly conserved regions of the mtDNA, and thus can be used across a wide variety of taxa and even phyla without the need for prior sequence knowledge (reviewed in Meyer, 1993). Overall, mtDNA has proven to be a very useful and popular tool in the elucidation of questions regarding hybrid zones, gene flow (via females), population structure and phylogeny (Moritz *et al.*, 1987; Avise, 1994; but see Rand, 1994).

6.1.3. Parentage analysis using RAPDs

The use of the RAPD technique in certain areas of study has received criticism for some of its shortcomings (reviewed in Black, 1993; Bachmann, 1994; Grosberg *et al.*, 1996). However, in the area of one-family parentage analysis it has generally been regarded with favour (Scott *et al.*, 1992; Majerus *et al.*, 1996). This is largely due to the ease with which large numbers of primers can be screened to identify diagnostic parental bands, at relatively low cost. Nonetheless, problems have been encountered where non-parental bands occur in the profiles of offspring (Riedy *et al.*, 1992). This problem may be circumvented if analysis is limited to only diagnostic bands (Scott *et al.*, 1992). The use of RAPDs in parentage analysis is consequently becoming commonplace for plants (Åkerman *et al.*,1995; Ahmad *et al.*, 1996; Parani *et al.*, 1997; Grashof-Bokdam *et al.*, 1998) and animals (Levitan &

Grosberg, 1993; Jones *et al.*, 1994; Bishop *et al.*, 1996; Stott *et al.*, 1997; Tirado & Lewis, 1997; Coffroth & Lasker, 1998).

6.1.4. *Celleporella hyalina*

Considerable study has been undertaken on the classification of the Hippothoidae (Ryland & Gordon, 1977; Hastings, 1979; Ryland, 1979; Morris, 1980; Moyano & Gordon, 1980). Although *Celleporella hyalina* is now treated as a distinct form, it remains synonymous with the genus *Hippothoa* (Hastings, 1979). Within the Hippothoidae, *C. hyalina* may be distinguished by a schizoporelloid ancestrula which buds asymmetrically with a spiral early astogeny, a pluriserial and bilaminar colonial form, with the majority of the polymorphic sexual zooids being frontally budded.

Despite the low dispersal potential of *C. hyalina* (see Chapter 5), it appears to occupy a circumglobal distribution in the colder seas of the Northern and Southern hemispheres (Marcus, 1938; Ryland & Gordon, 1977; Hastings, 1979; Ryland, 1979; Morris, 1980; Moyano, 1986). This extensive range may, in part, be due to isolated incidents of long distance dispersal by rafting (Knowlton & Jackson, 1993). Epiphytic bryozoans have been found to have significantly wider ranges than species attached to more permanent substrata (Watts *et al.*, 1998). Such isolated colonisation events are likely to result in rapid differentiation of the founder population. If further gene flow is sufficiently restricted, speciation eventually occurs (Slatkin, 1996). It might be expected that populations of *C. hyalina* occupying different oceanic basins are sufficiently isolated for speciation to occur. The frequency of cryptic or sibling species amongst widespread bryozoan taxa is thought to be considerable (Lidgard & Buckley, 1994).

Over its circumglobal distribution *C. hyalina* occurs as a relatively common intertidal bryozoan on the shores of Chile (Moyano, 1986; J. Cancino pers. comm.) and Britain (Ryland & Hayward, 1977). Previous studies of life history traits of Welsh and Chilean *C. hyalina* populations, have yielded differing results, indicating potentially profound ecological differences between the two. For example, larvae released from Chilean colonies have been found to undergo settlement and metamorphosis even after 28 hours of enforced swimming. Welsh larvae, in contrast, are unable to metamorphose if forced to swim for longer than 4 hours (Orellana & Cancino, 1991; Orellana *et al.*, 1996). Chilean colonies reared in isolation produce

only male sexual zooids (Cancino *et al.*, 1991), whereas Welsh colonies grown without conspecifics produce both male and female zooids (Hunter & Hughes, 1993a; P. Manríquez pers. comm.). Field populations from the two countries appear to occupy different ecological niches. Chilean *C. hyalina* is predominantly found associated with holdfasts of *Macrocystis integrifolia* or attached to rocks (Cancino *et al.*, 1991; J. Cancino pers. comm.). Welsh *C. hyalina*, although highly catholic in its choice of substrata, is most prominent on the fronds of *Laminaria saccharina* and *Fucus serratus* (Hayward & Ryland, 1979; Cancino, 1986). Assigning identity to this widely dispersed species may give some indication of the likelihood of potential cryptic speciation in other similar marine invertebrates. Knowlton (1993) argues that the failure to recognise cryptic species in the marine environment, cripples the ecological and evolutionary understanding of the oceans.

6.2. MATERIALS AND METHODS

6.2.1. Study Material

Colonies of *C. hyalina* were collected on small fragments of substrata from sites around the Atlantic basin and the western coast of South America (Fig. 6.1). Samples from a total of six sites were collected over a one year period (Table 6.1).

Table 6.1. Sites of *C. hyalina* sample collection.

Country	Area	Substrate	Longitude & Latitude
Wales	Menai Strait, Gwynedd	*Laminaria saccharina*	53° 13' N 04° 11' W
England	Yealm Estuary, Devon	*Laminaria saccharina*	50° 17' N 04° 11' W
Sweden	Tjärnö, Göteborg	*Laminaria saccharina*	58° 54' N 11° 07' E
U. S. A.	Damariscotta Estuary, Maine	*Laminaria longicruris*	43° 54' N 69° 43' W
Chile	Las Cruces, Valparaíso	*Macrocystis integrifolia*	33° 30' S 71° 38' W
Chile	Concepción, Bíobío	Rock	36° 50' S 75° 45' W

Approximately ten colonies were collected from each site. Samples were preserved in 70 % ethanol and stored, where possible at 4 °C. Colonies were removed from the adherent substratum with a rounded scalpel blade, before DNA extraction. In addition to the ethanol preserved samples, 10 live colonies were brought from Las Cruces, Chile on a shard of plate glass. These colonies were maintained in 2 litre cylindrical polyethylene containers, part-filled with 0.2 µm filtered UV-irradiated sea water and a suspension of *Rhinomonas reticulata* at a concentration of 100 cells μl^{-1}.

6.2.2. Cross fertilisation experiments

The 10 live Chilean *C. hyalina* colonies were subjected to a period of 24 hours total darkness then exposed to bright illumination in order to facilitate larval release (Ryland, 1959). Upon release, larvae were carefully pipetted into circular 400 ml plastic vessels containing 350 ml of 0.2 µm filtered UV-irradiated sea water, micro-algal diet and acetate sheets. The acetate sheets had previously been conditioned for 1 month in settled sea water to acquire a biofilm in order to enhance settlement (Hunter & Hughes, 1991). The containers were then placed in total darkness for 8 hrs. After this period the acetate sheets were examined for newly

settled non-feeding ancestrulae. The ancestrulae were individually cut from the acetate sheets and then placed in separate covered 300 ml plastic beakers, containing 250 ml of filtered sea water and mico-algal diet at a concentration of 100 cells μl^{-1}. Algal cells were kept in suspension via an airstone. Individual ancestrulae from Welsh colonies were settled in the above manner and placed in the same beaker as Chilean ancestrulae. The two pieces of acetate supporting 1 Welsh and 1 Chilean ancestrulae were held, back to back, with a short length (≈5 cm) of plastic spine binder. A total of 12 cross-treatments were set up. Four control beakers were prepared, two of which contained two Welsh ancestrulae and the remaining two contained two Chilean ancestrulae. Filtered sea water and algal suspension were changed daily. The growing colonies were cleaned with a fine artists' brush, weekly, taking care to avoid any cross-contamination of other cultures with sperm. These potential parental colonies were cultured for a period of two months. At the end of this period the number of autozooids, frontal male zooids, female zooids (ovicells) and embryos were counted using a Wild dissecting microscope. Regrettably, the growth of an algal film beneath the colony and abrasion of the acetate sheet, made accurate counts of the number of basal male zooids impossible, although presence was noted, where possible. Released larvae were allowed to settle and grow on the plastic vessels or acetate sheets. The newly settled colonies were counted and tissue samples taken for RAPD analysis.

6.2.3. DNA extraction and RAPD profiling

For protocols for both DNA extraction and RAPD profiling see chapter 5. Due to the possibility of self-fertilisation in *C. hyalina* (Hunter & Hughes, 1993a), it was deemed necessary to obtain RAPD profiles for any offspring produced in cross-fertilisation treatments. In this way, actual incidence of successful cross-fertilisation and production of competent larvae could be ascertained.

6.2.4. Mitochondrial DNA sequencing

The amplification of the cytochrome oxidase subunit I (COI) portion of the mitochondrial genome follows a modified method of Folmer *et al.*,(1994).

Amplification reactions were conducted in 1 X PCR Buffer (20 mM Tris HCl pH 8.4, 50 mM KCl), $MgCl_2$ (2.5 mM) and 1.0 unit of Taq DNA polymerase.

Primers used were those of Folmer *et al.*, (1994) at a concentration of 280 nM. All primers and reagents were supplied by Gibco BRL. Finally, 0.5 µl of DNA suspension at a concentration of 60 ng μl^{-1} was added. The reaction mixture was overlaid with an equal volume of mineral oil and amplified in an OmniGene thermal cycler (Hybaid, UK) programmed as given in Table 6.2.

Table 6.2. Thermal cycler amplification conditions.

Stage	Function	Temperature (°C)	Time (Mins.)	No. Cycles
1	Denature	93	1.00	10
	Annealing	47	2.00	
	Extension	72	2.00	
2	Denature	93	1.00	25
	Annealing	47	2.00	
	Extension	72	3.00	
3	Final Ext.	72	5.00	1
	Room Temp.	28	0.50	

Amplification was confirmed and products purified using gel electrophoresis. The PCR products were electrophoresed at 120 Volts for 1 Hour on 1.2 % agarose gels with 1 X TBE buffer (0.089 m Tris borate, 0.002 m EDTA) and stained with Ethidium bromide. Gels were visualised with UV illumination. Product bands were excised and subjected to purification using GenElute agarose spin columns. The purified segment of mtDNA was then sequenced directly (Winship, 1989) using the Sequenase Version 2.0 DNA sequencing kit. Resulting sequences were aligned by eye. A sample from each site was also commercially sequenced (Alta Bioscience, Birmingham) to verify manually obtained sequences. Three individuals were sequenced from each site. In all cases sequences were found to be invariant within sites.

6.2.5. Statistical analysis

Data from observations of growth and reproductive allocation of potential parental Welsh and Chilean colonies were subjected to the Anderson-Darling test to assess the normality of the data obtained, then analysed accordingly (Minitab, 1996). Two-sample *t*-tests were employed unless data were found not to be normally distributed, in which case Mann-Whitney two-sample rank tests were performed (Zar, 1984).

Polaroid photographs of RAPD profile gels were scored in a highly conservative manner. Consistently faint or unrepeatable bands were excluded in order to eliminate false results. RAPD bands of a specific size were scored for presence or absence. A genetic distance matrix based on band sharing was calculated with unweighted pair-group mean arithmetic average (UPGMA) cluster analysis (Sneath & Sokal, 1973) and results displayed as a dendrogram and multi-dimensional scaling scatterplots using the STATISTICA package (StatSoft, Inc.1995).

Parentage was assigned by two methods. Firstly, bands were identified that occurred in the offspring and allowed one of the parents to be excluded (exclusion). Secondly, bands unique to the offspring and one parent were identified (inclusion) (Levitan & Grosberg, 1993) and results were tabulated (Table 6.6).

Sequence data were subjected to both discrete character and distance based tree construction methods. Maximum parsimony (MP) and maximum likelihood (ML) methods are both discrete character based. MP builds a tree using the fewest evolutionary steps (base substitutions in this instance) to explain the data obtained. The most parsimonious tree was identified via a heuristic search using the PAUP package (Swofford, 1993). ML methods evaluate the probability of a specified evolutionary model giving rise to the data observed. Phylogeny can then be inferred by identifying trees with the highest likelihood (Swofford *et al.*, 1996). The PHYLIP phylogeny inference package (Felsenstein, 1993) was used to carry out the ML method. The distance based method of tree construction used was Neighbour-joining (N-J)(Saitou & Nei, 1987). Such distance based methods require the formation of OTU x OTU (Operational Taxonomic Unit) matrices. Matrices were constructed using the Kimura two-parameter model of nucleotide substitution (Kimura, 1980) using the MEGA package (Kumar *et al.*, 1993). The Kimura model may underestimate divergence times if sequences are highly variable, due to mutational saturation at the most changeable third codon position. Consequently the amount of substitutional saturation was assessed graphically by plotting transition and transversion substitutions against the Kimura distance. The Kimura model does have the advantage of being particularly suited to the formation of matrices for closely related OTUs. This is because the model adjusts for a higher rate of transitions than transversions, which may be common in closely related organisms (Irwin *et al.*, 1991).

Figure 6.1. Site map of coastal sampling areas in the Atlantic basin and South Eastern Pacific.
■ = Wales, Menai Strait, ★ = England, Devon, ✚ = Sweden, Göteborg, ◆ = USA, Maine, ▲ = Chile, Las Cruces, ● = Chile, Concepción.

6.3. RESULTS

6.3.1. Growth and reproductive allocation in Chilean and Welsh colonies

Irrespective of treatment, a marked difference in the number of autozooids produced was observed between Chilean and Welsh parental colonies (Tables 6.3 & 6.4). Welsh colonies had, on average, approximately four times more autozooids than Chilean colonies (Fig. 6.2). Chilean colonies exhibited significantly more frontal males per autozooid in the controls than in the cross treatments (Fig. 6.3; Table 6.3). In contrast Welsh colonies showed the most frontal males per autozooid in the cross treatments (Fig. 6.3; Table 6.3). Basal male zooids were noted to occur in colonies from both populations, in all treatment groups. No significant difference was detected between the number of female zooids (ovicells) per autozooid between control and cross treatments for Welsh colonies, or between Welsh and Chilean colonies in the control treatment (Tables 6.3 & 6.4; Fig. 6.4). Chilean colonies in the cross treatments failed to produce any female zooids (Fig. 6.4). Welsh control colonies had a significantly larger number of embryos per female than Welsh colonies in cross treatments (Mann-Whitney W = 85.0, P = <0.05, n = 12, n = 4). However, no significant difference was detected between Chilean and Welsh control colonies for number of embryos per female (Mann-Whitney W = 15.0, P = 0.4705, n = 12, n = 4)(Fig. 6.5).

Table 6.3. Results of two sample *t*-tests for controls compared with cross treatment colonies separated by origin of colonies. Sexual zooids are considered as a proportion of autozooids within the colony.

	CHILEAN			WELSH		
	T	*df*	*P*	*T*	*df*	*P*
Autozooids	-3.77	4	0.020*	6.51	8	<0.001*
Males	-2.82	9	0.020*	2.66	13	0.020*
Females	--	--	--	2.04	8	0.076

T = t value, *df* = degrees of freedom (calculated from the variances of the two samples and truncated to the nearest integer), *P* = P value, * = significant values, -- = no females produced.

Table 6.4. Results of two sample *t*-tests for Welsh colonies compared with Chilean colonies in control and cross treatments. Sexual zooids are considered as a proportion of autozooids within the colony.

	CONTROL			CROSS		
	T	*df*	*P*	*T*	*df*	*P*
Autozooids	3.54	4	0.024*	-7.59	20	<0.001*
Males	1.78	4	0.150	-3.46	18	0.002*
Females	0.18	4	0.870	--	--	--

T = t value, *df* = degrees of freedom, *P* = P value, * = significant values, -- = no females produced.

Observations made using both light and scanning electron microscopy of Chilean and Welsh colonies grown in the experimental set-up show differences in zooidal morphology (Figs. 6.6 to 6.9). The most obvious difference was observed in the increased number of tubular pore chambers in the inter-zooidal region, Chilean colonies generally possessing 14-16 pores compared with the 6 pores found in the Welsh specimens (n=10 and n=100 respectively). The autozooids of Chilean colonies also appeared to have a less convex distal surface than the autozooids of Welsh colonies.

6.3.2. RAPD analysis of cross-treatment progeny

Welsh control treatments contained a total of 319 settled larvae (228 in pot WW1 and 91 in WW2). No settled larvae were observed in the Chilean control pots. Only 2 out of the 12 cross-fertilisation treatments containing both Chilean and Welsh colonies produced any larvae which settled. The number of larvae observed to have settled in these 2 treatments was 3 and 7 respectively. A total of 89 bands were scored from RAPD profiles of the potential parents and larvae found in the two cross-fertilisation treatments (Table 6.5). These offspring exhibited no bands found in profiles of the potential parental Chilean colonies (Fig. 6.10). From the larvae produced in each of the 2 cross fertilisation treatments a non-parental band was observed. One of these was not observed in any of the other profiles produced, while the other was common to a band in the Welsh parent from the other treatment. The minimum number of 50 loci to be scored for each offspring, suggested by Lewis &

Snow (1992) for parentage analysis, is exceeded but probably unnecessary owing to the unambiguous nature of the results.

Table 6.5. Number of monomorphic and polymorphic bands scored from RAPD profiles, for both potential parental colonies.

	Primer 5		*Primer 6*		*Primer 7*		*Primer 8*		*Primer 9*	
	M	*P*	*M*	*P*	*M*	*P*	*M*	*P*	*M*	*P*
Chilean	4	5	5	6	6	3	2	1	3	2
Welsh	4	4	6	6	6	6	2	5	3	10

Data presented are, M = number of monomorphic bands, P = number of polymorphic bands.

UPGMA cluster analysis is displayed as a dendrogram of percentage disagreement for offspring and potential parents from both cross treatment containers which yielded larvae (Fig. 6.11). Multi-dimensional scatterplots are also presented for offspring and potential parents from separate containers (Figs. 6.12 & 6.13). The genotypes of the offspring are considerably more similar to that of the respective Welsh colony present than that of the Chilean colony. However, the scatterplots show that the progeny do differ genetically, albeit to a lesser degree, from the Welsh parental colony.

Assignment of parentage by both exclusion and inclusion yielded identical results. Marker bands present in the offspring which enabled the exclusion of one potential parent (Chilean) were always common to the Welsh parent and thus could be assigned to that parent by inclusion (Table 6.6). A mean of 31.5 ± 3.77 bands identified the Welsh colony as the parent, in both inclusion and exclusion, for all 10 offspring.

Table 6.6. Assignment of parentage by exclusion and inclusion. Values indicate the number of loci that are absent in the parental profile (for exclusion), or are unique to one parent and that offspring (for inclusion).

	PATERNITY BY EXCLUSION		PATERNITY BY INCLUSION	
Offspring	*Welsh Parent*	*Chilean Parent*	*Welsh Parent*	*Chilean Parent*
A1	0 *	35	35 *	0
A2	0 *	33	33 *	0
A3	0 *	35	35 *	0
A4	0 *	33	33 *	0
A5	0 *	32	32 *	0
A6	0 *	35	35 *	0
A7	0 *	33	33 *	0
B1	0 *	28	28 *	0
B2	0 *	25	25 *	0
B3	0 *	26	26 *	0

*, indicates most likely parent

6.3.3. Sequence data

Sequencing of the mtDNA COI provided mitochondrial haplotypes of 365 bp for *C. hyalina* samples from all 6 sites, plus *Electra pilosa* as an outgroup (Appendix 3.1). Plots of the Kimura genetic distance against numbers of transitions and transversions were linear, indicating no saturation effect (Fig 6.14 & 6.15). Trees were obtained using ML (Fig. 6.16), N-J (Fig. 6.17) and MP (Fig. 6.18). The heuristic search for the most parsimonious trees, obtained a single tree with a length of 216 steps (Fig. 6.18). The branching patterns produced by the different tree building methods show a relatively high degree of similarity, implying that this is a generally robust topology. However, some variation in node position is evident in the MP tree with regard to the Las Cruces and Concepción samples. Tree topology correlates well with geographic distance, the Chilean specimens being the most different from the Welsh specimens (excluding the *E. pilosa* outgroup). Proportion of sequence divergence is overall extremely high (Table 6.7.). The Welsh and English samples were most similar, while the Swedish and Chilean (Concepción) samples were most contrasting. Although, more similar than most, a relatively high divergence was found between the two Chilean samples.

Table 6.7. Table of proportion of sequence divergence for *C. hyalina* samples.

	Wales	England	Sweden	U.S.A.	Chile, LC.	Chile, Con.
Wales		0.0496	0.2176	0.2176	0.2314	0.2645
England	--		0.2039	0.2066	0.2424	0.2755
Sweden	--	--		0.2011	0.2590	0.2782
USA	--	--	--		0.2149	0.2590
Chile, LC.	--	--	--	--		0.1019
Chile, Con.	--	--	--	--	--	

Chile, LC. = Las Cruces, Chile, Con. = Concepción.

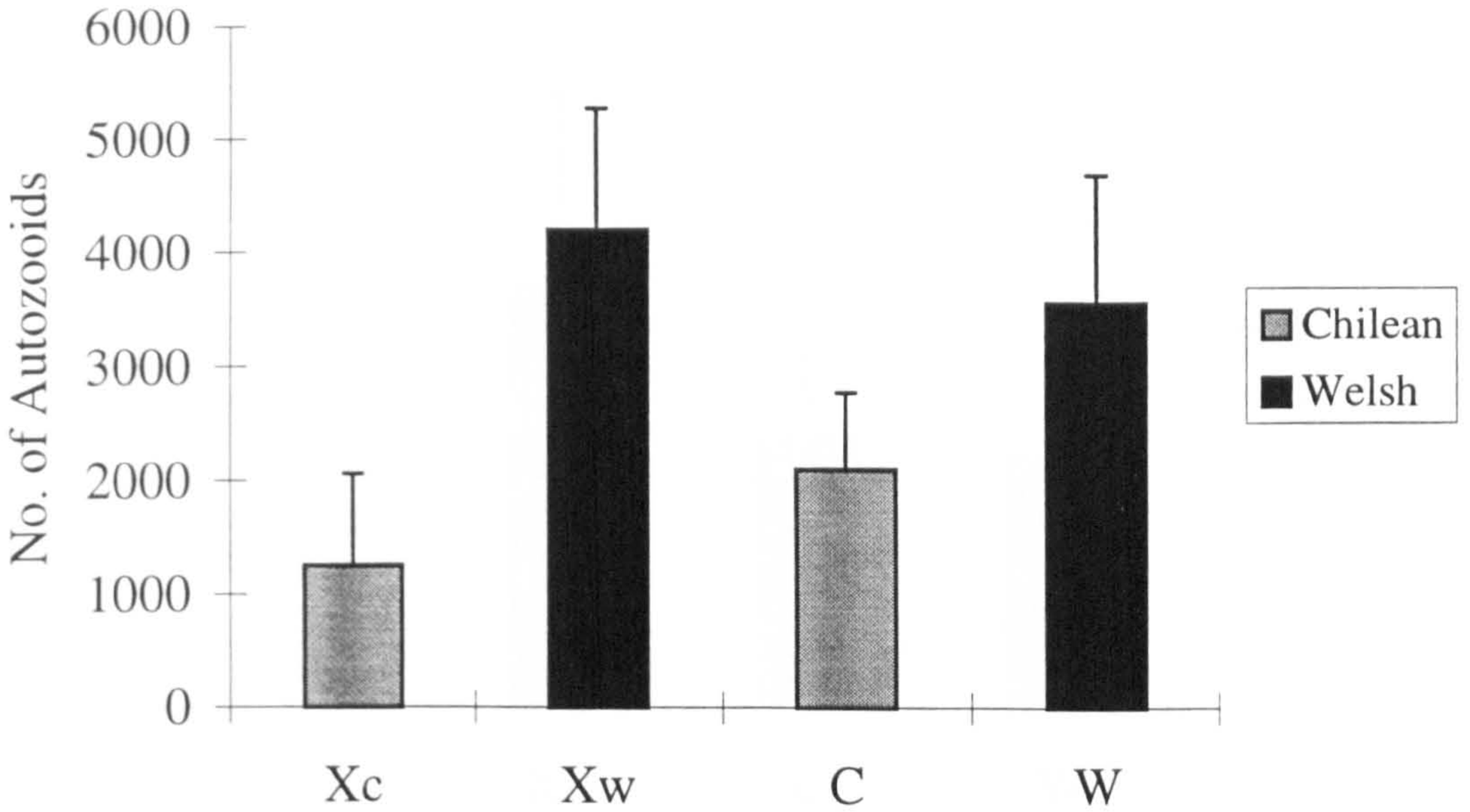


Figure 6.2. Number of autozooids (Mean + 1SD) observed in control and Chilean Welsh cross treatments after 56 days. Xc = Chilean cross treatment colonies (n=12), Xw = Welsh cross treatment colonies (n=12), C = Chilean control (n=4), W = Welsh control (n=4).

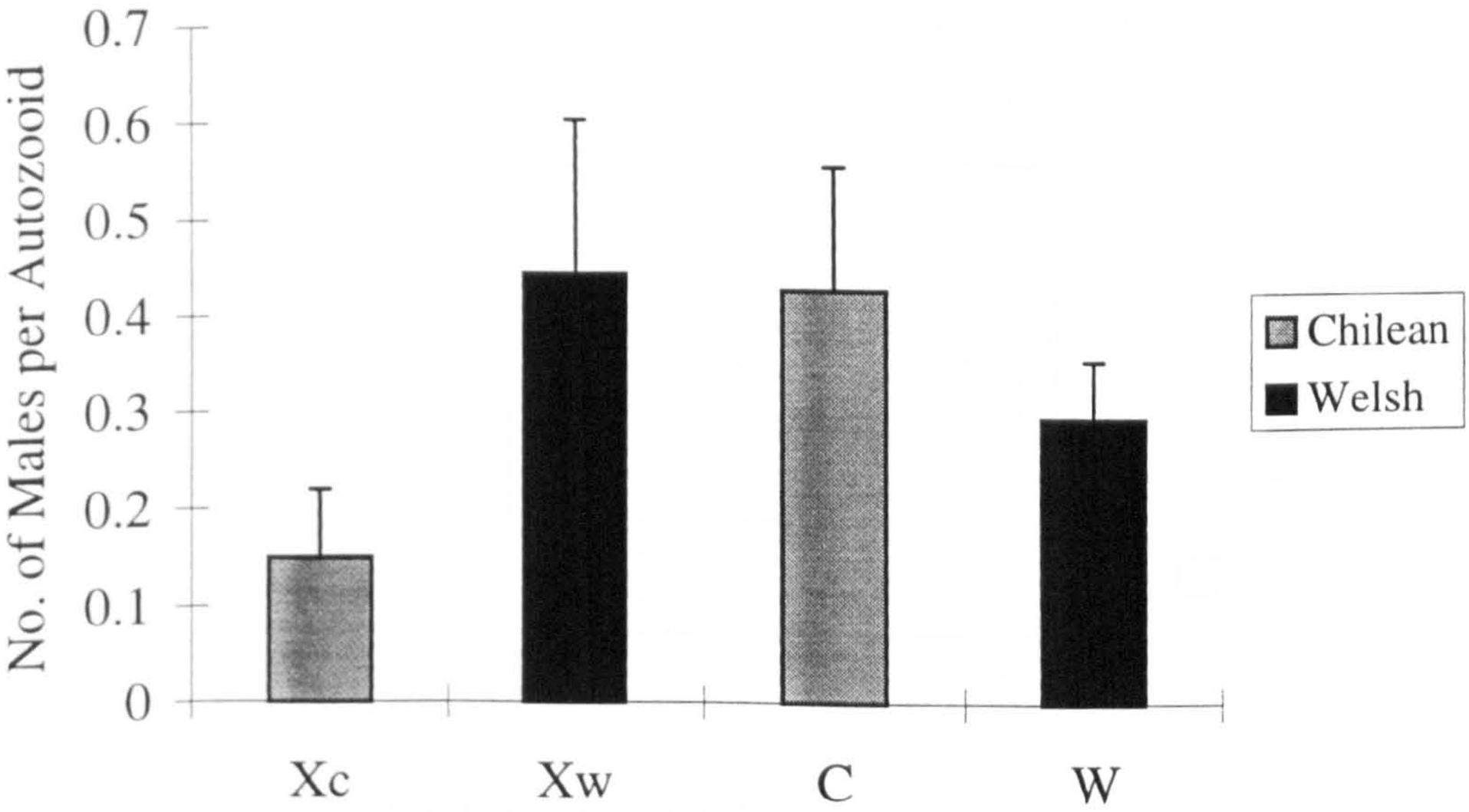


Figure 6.3. Number of male zooids per autozooids (Mean + 1SD) observed in control and Chilean Welsh cross treatments after 56 days. Xc = Chilean cross treatment colonies (n=12), Xw = Welsh cross treatment colonies (n=12), C = Chilean control (n=4), W = Welsh control (n=4).

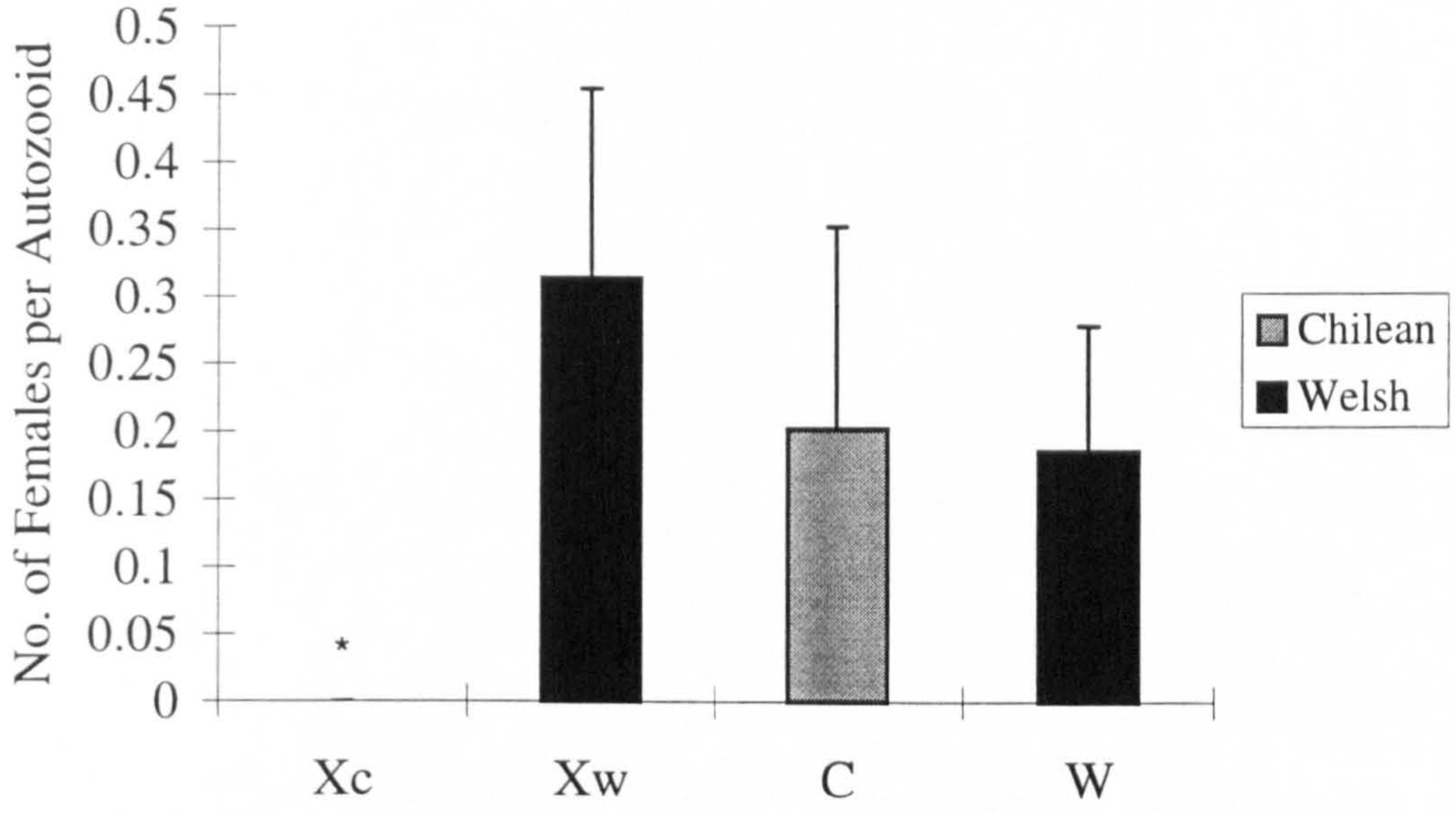


Figure 6.4. Number of female zooids (ovicell) per autozooids (Mean + 1SD) observed in control and Chilean Welsh cross treatments after 56 days. Xc = Chilean cross treatment colonies (n=12), Xw = Welsh cross treatment colonies (n=12), C = Chilean control (n=4), W = Welsh control (n=4). * = No female zooids present.

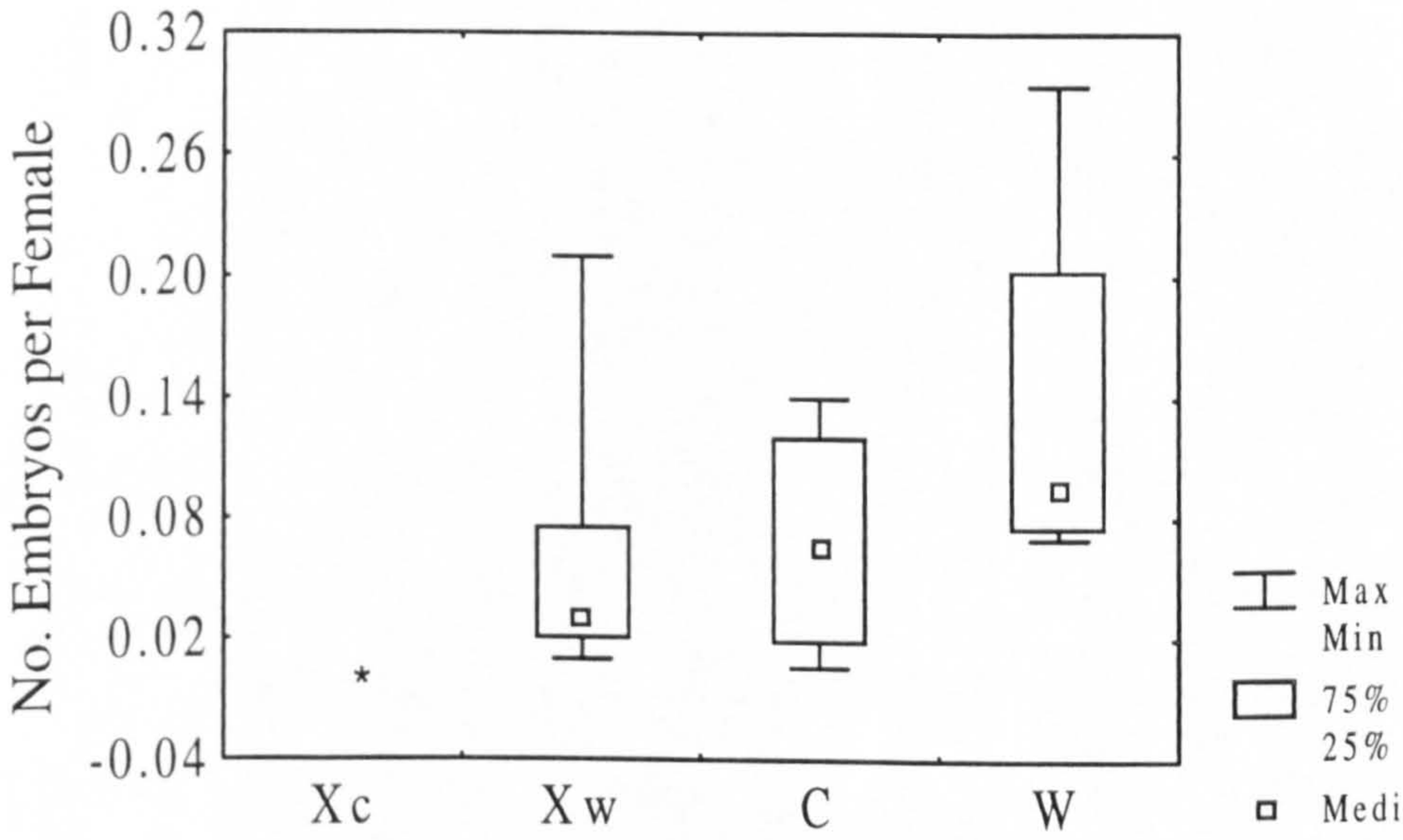


Figure 6.5. Number of embryos per female zooid (ovicell) (Medians, upper and lower quartiles, minimum and maximum values) observed in control and Chilean Welsh cross treatments after 56 days. Xc = Chilean cross treatment colonies (n=12), Xw = Welsh cross treatment colonies (n=12), C = Chilean control (n=4), W = Welsh control (n=4). * = No female zooids present. 75% to25% boxes define upper and lower quartiles.

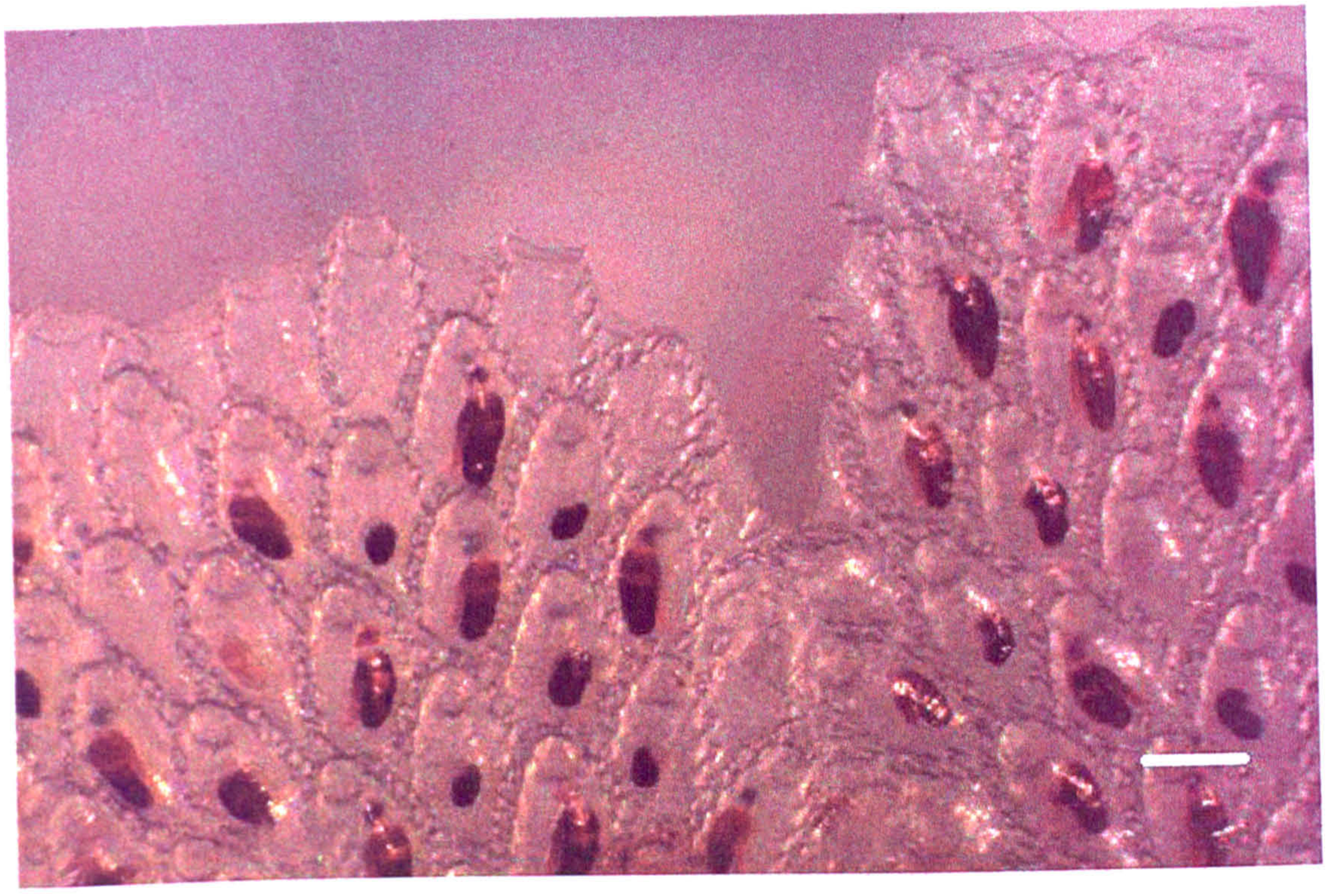


Figure 6.6. Colony of Chilean *C. hyalina*, showing autozooids and basal male zooids. Scale bar = 200 μm.

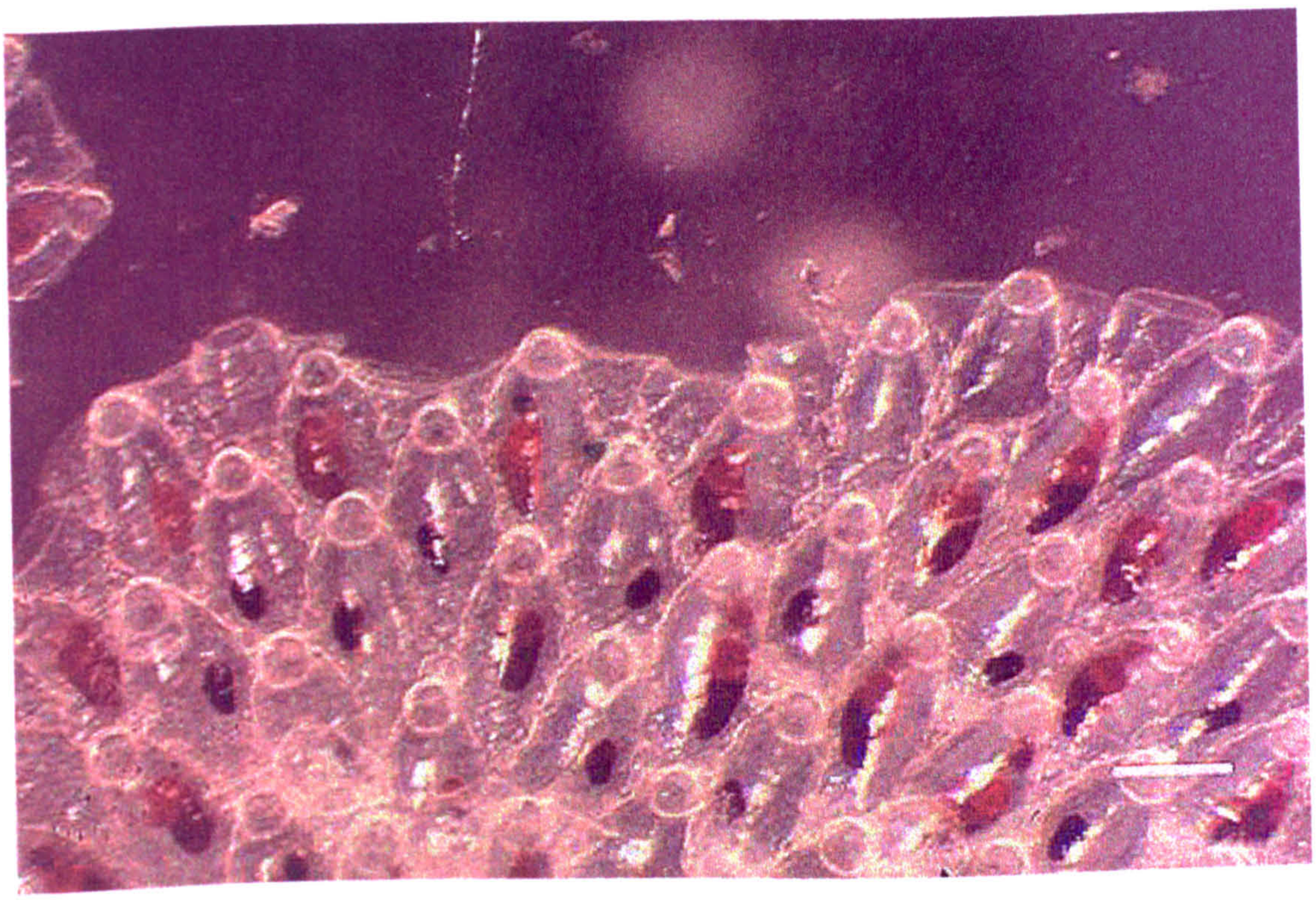


Figure 6.7. Colony of Welsh *C. hyalina*, showing autozooids. Scale bar = 200 μm.

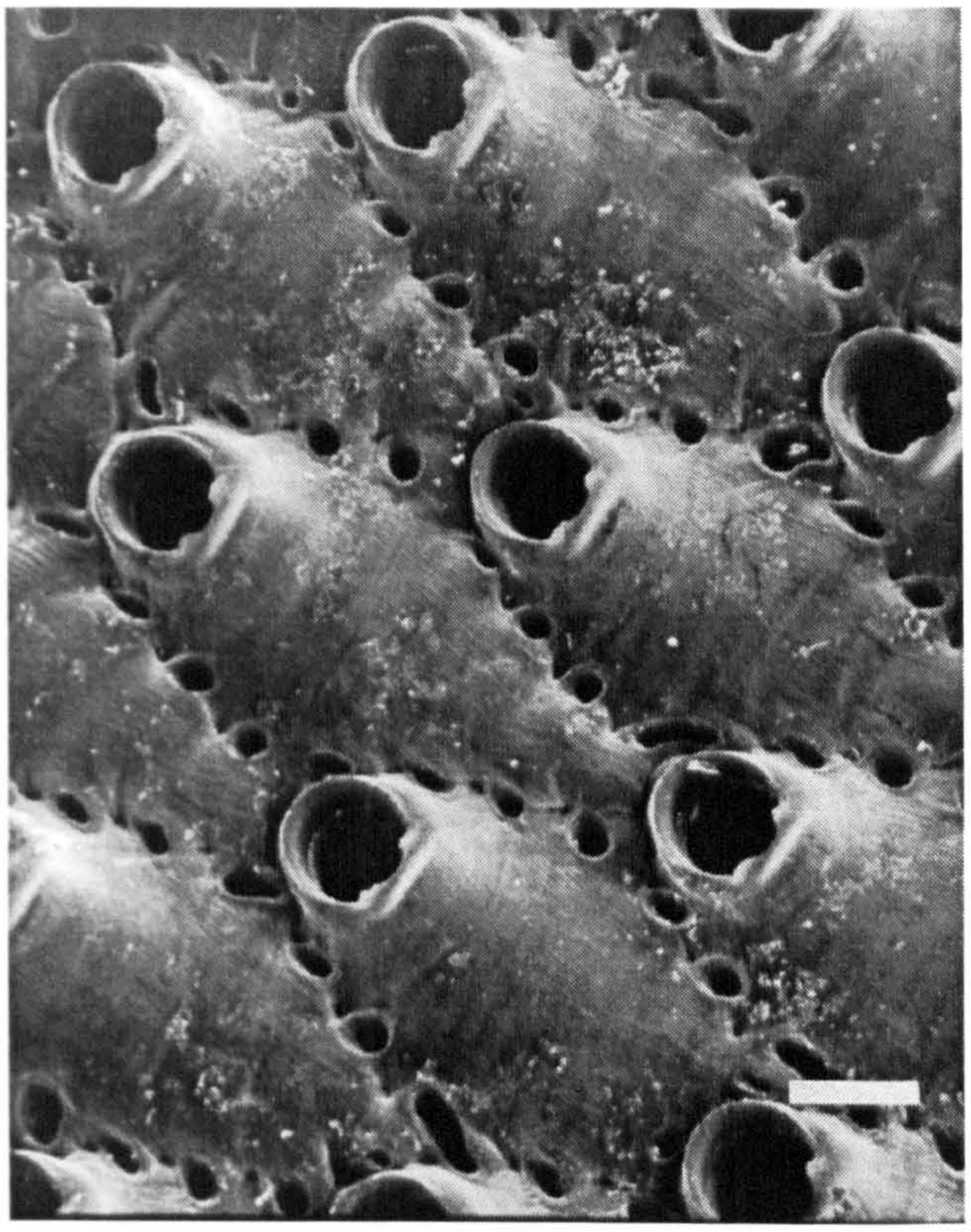


Figure 6.8. Scanning electron micrograph of autozooids from a Chilean colony, exhibiting tubular pore chambers. Scale bar = 100 μm

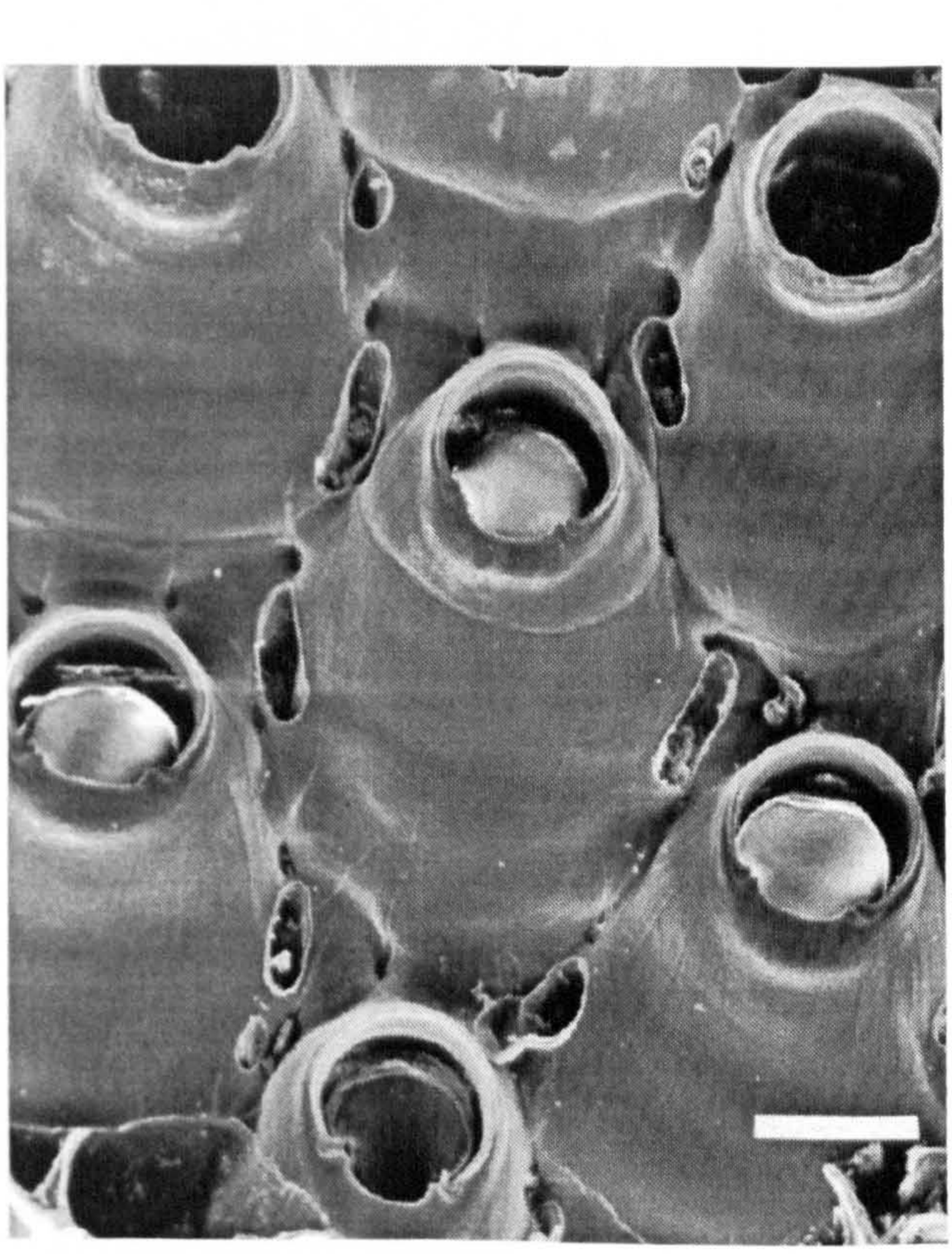


Figure 6.9. Scanning electron micrograph of autozooids from a Welsh colony, exhibiting tubular pore chambers. Scale bar = 100 μm.

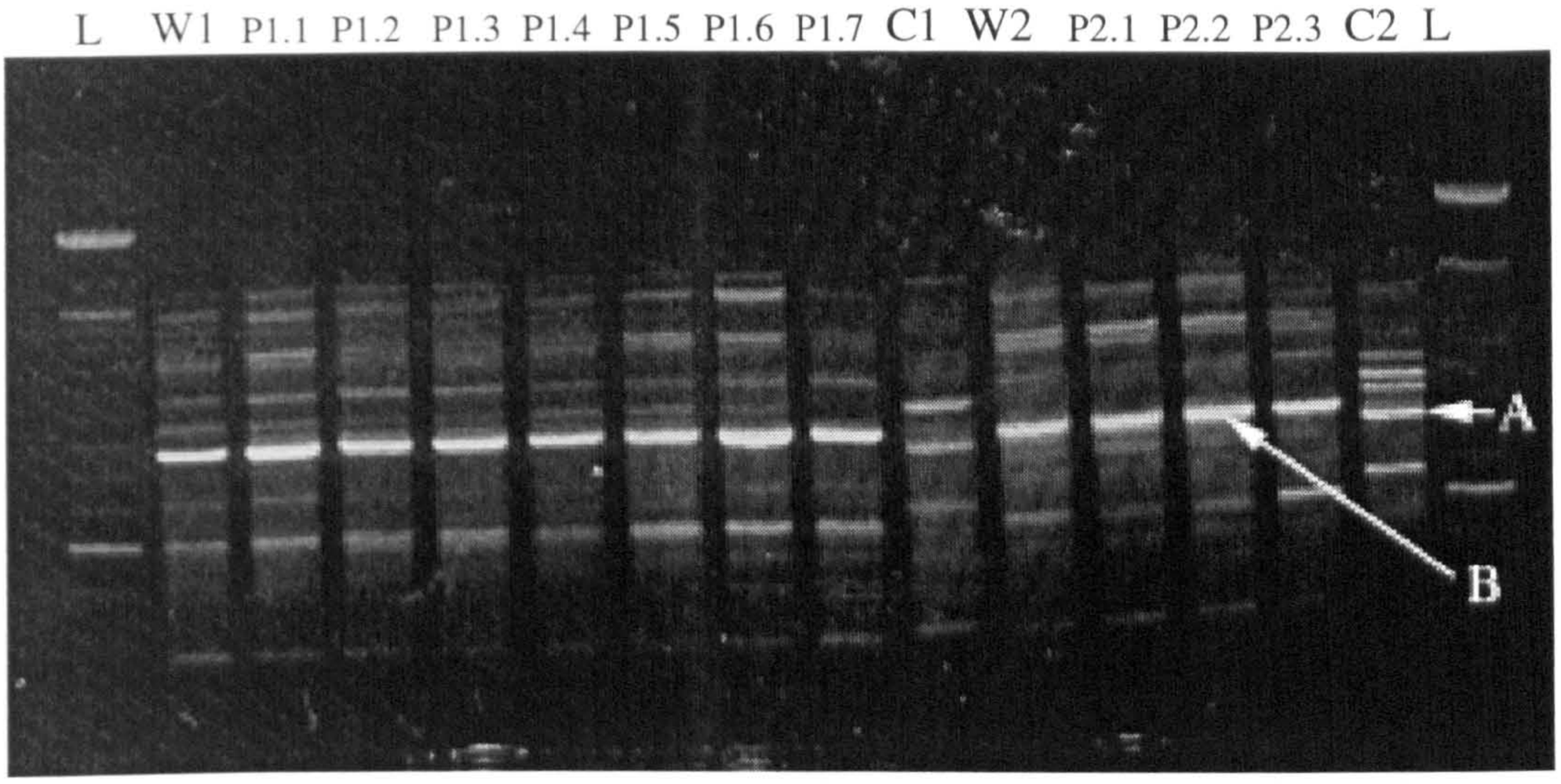


Figure 6.10. Photograph of RAPD profiles obtained using primer 8 for potential parents and progeny of cross-fertilisation treatments which produced offspring. L = 100 bp ladder, W = Welsh parental profile, C = Chilean parental profile, P = offspring profile, First number associated with letter indicates pot, second number used to identify offspring.

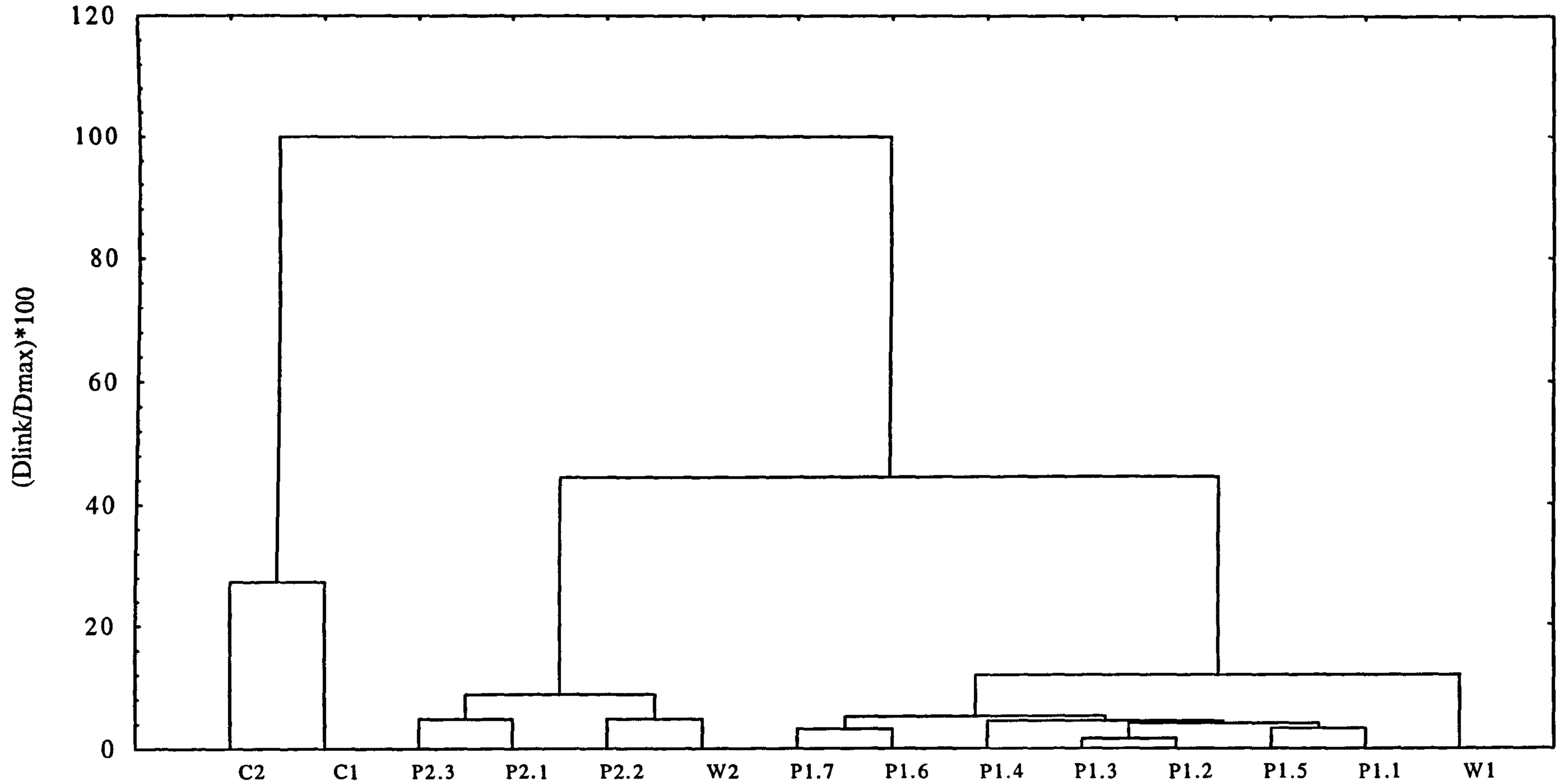


Figure 6.11. UPGMA dendrogram (percentage disagreement) for RAPD data for potential parents and resulting progeny from cross-treatments (1 & 2).

C = Chilean colony, W = Welsh colony, P = Progeny. Progeny second digit = Progeny number. (Dlink/Dmax)*100 = Distance expressed in terms of the largest observed distance.

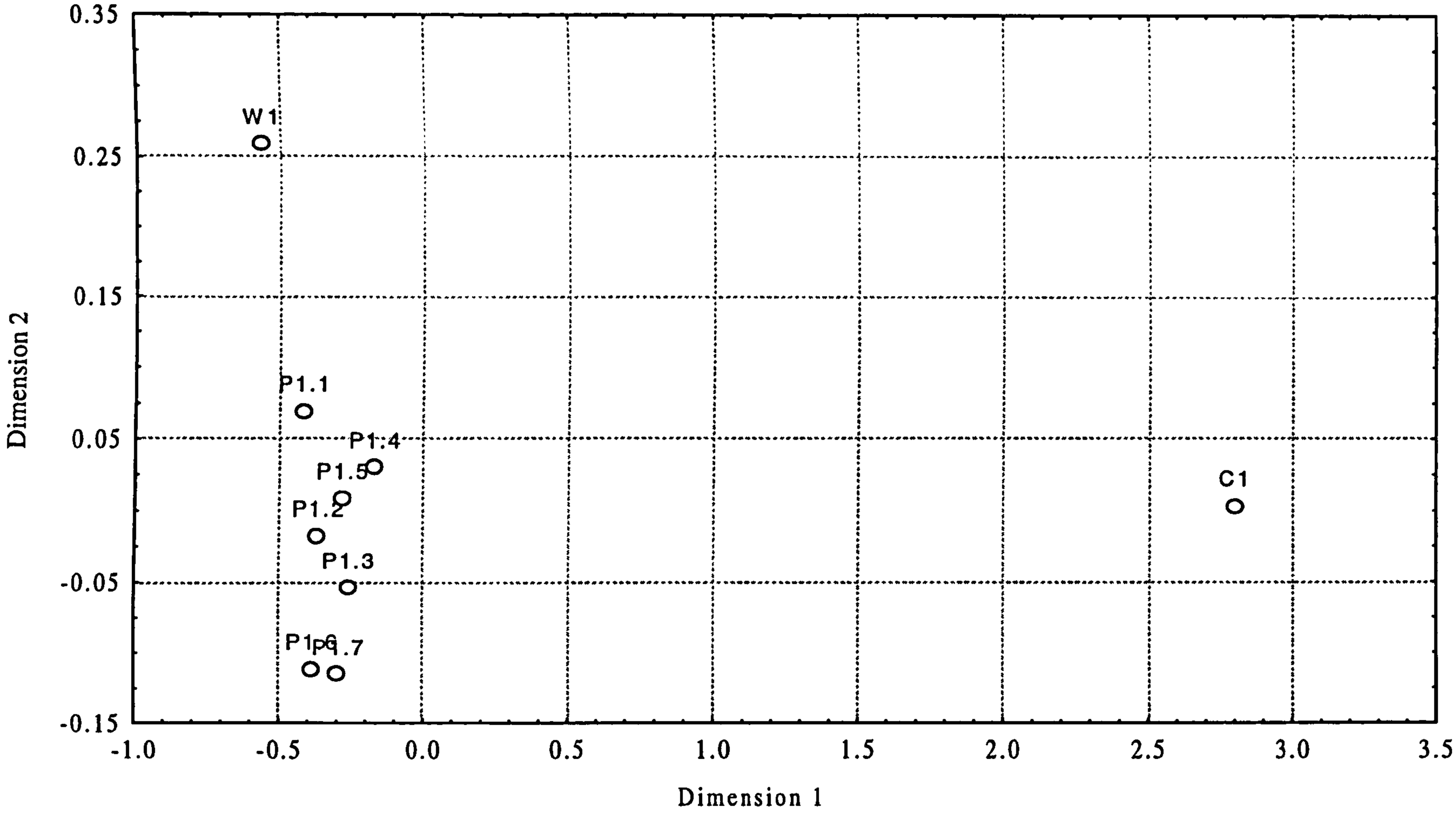


Figure 6.12. Multi-dimensional scaling scatterplot from RAPD data for potential parents and offspring from cross-treatment 1.
W1 = Welsh colony, C1 = Chilean colony, P1.1 to P1.7 = Progeny.

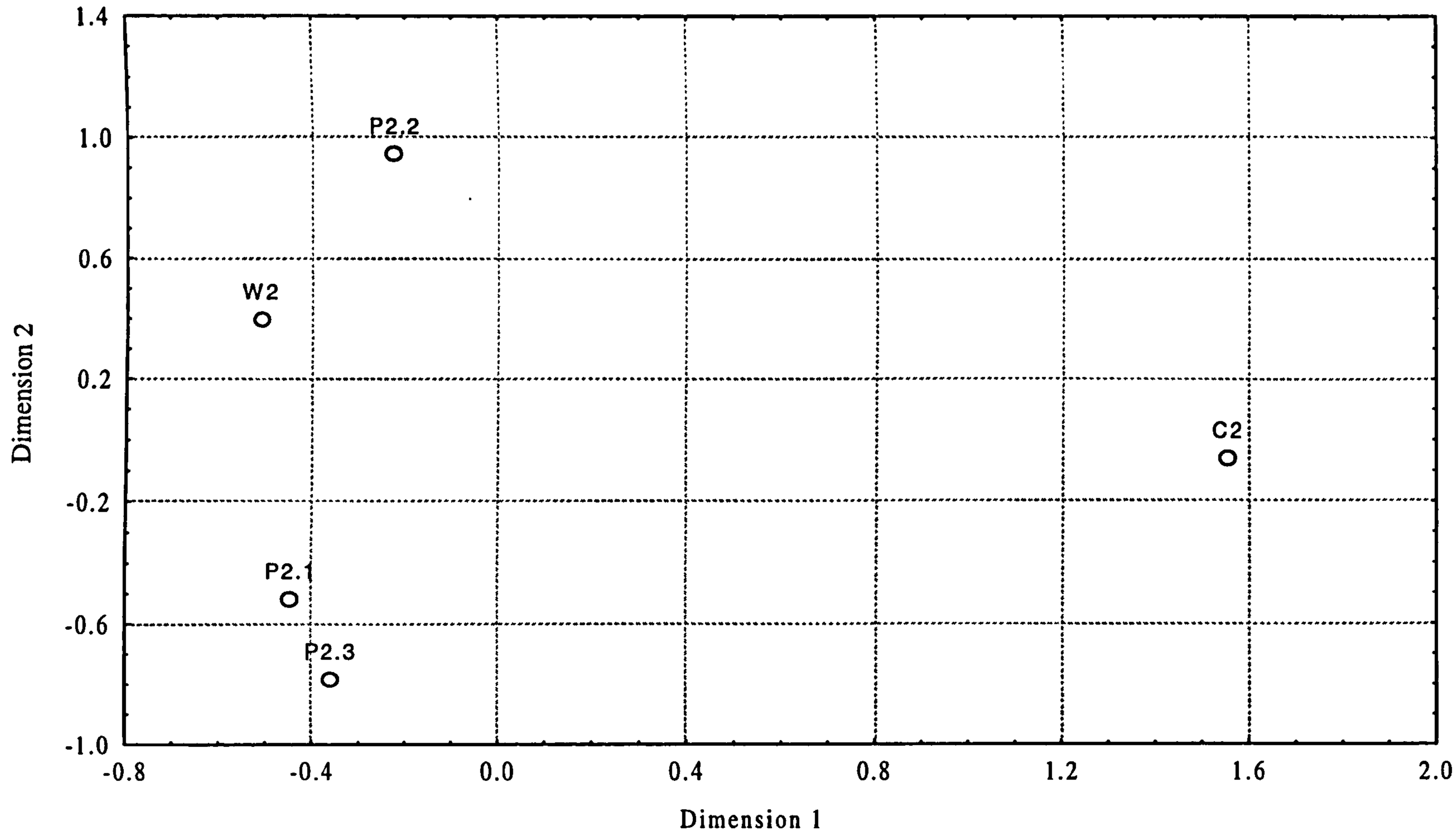


Figure 6.13. Multi-dimensional scaling scatterplot from RAPD data for potential parents and offspring from cross-treatment 2.
W2 = Welsh colony, C2 = Chilean colony, P2.1 to P2.3 = Progeny.

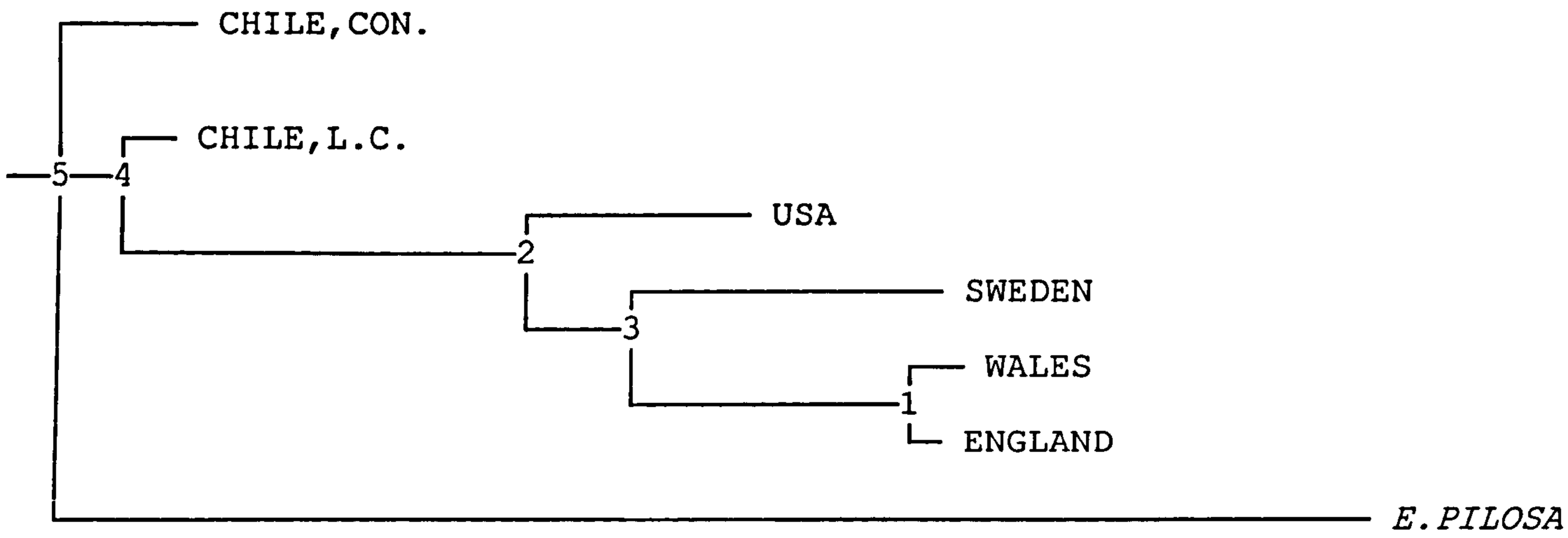
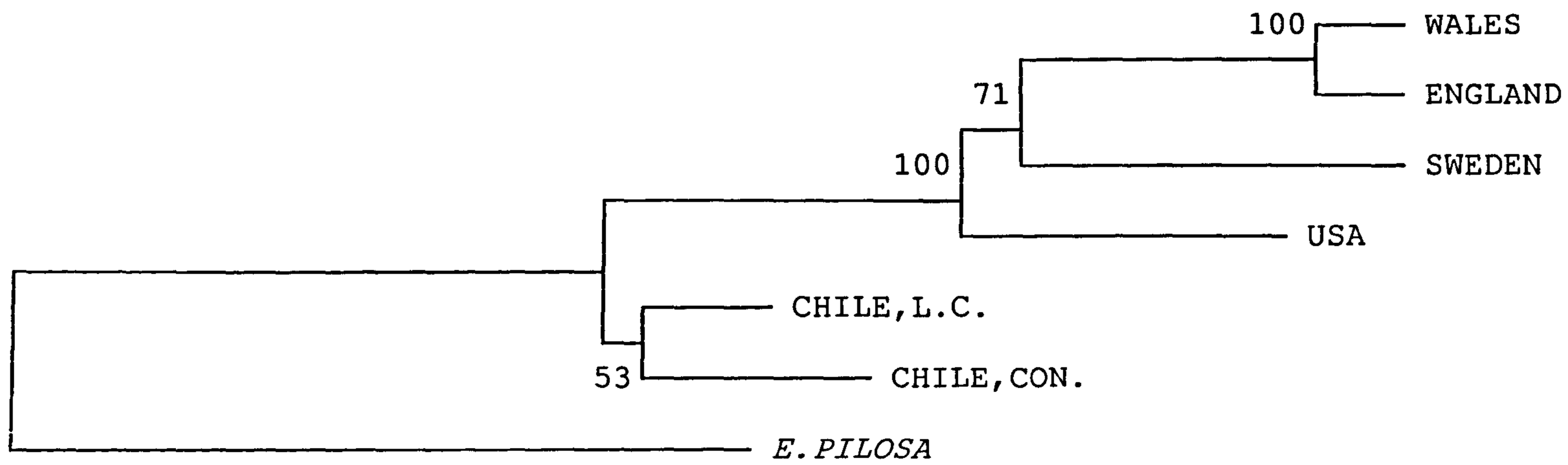


Figure 6.16. Phylogenetic tree formed using maximum likelihood. Ln Likelihood = -1865.80804. Confidence limits (Appendix 3.2).



Scale: each - is approximately equal to the distance of 0.006471

Figure 6.17. Neighbour Joining tree. Numbers associated with branches represent bootstrap values derived from 500 replicates.

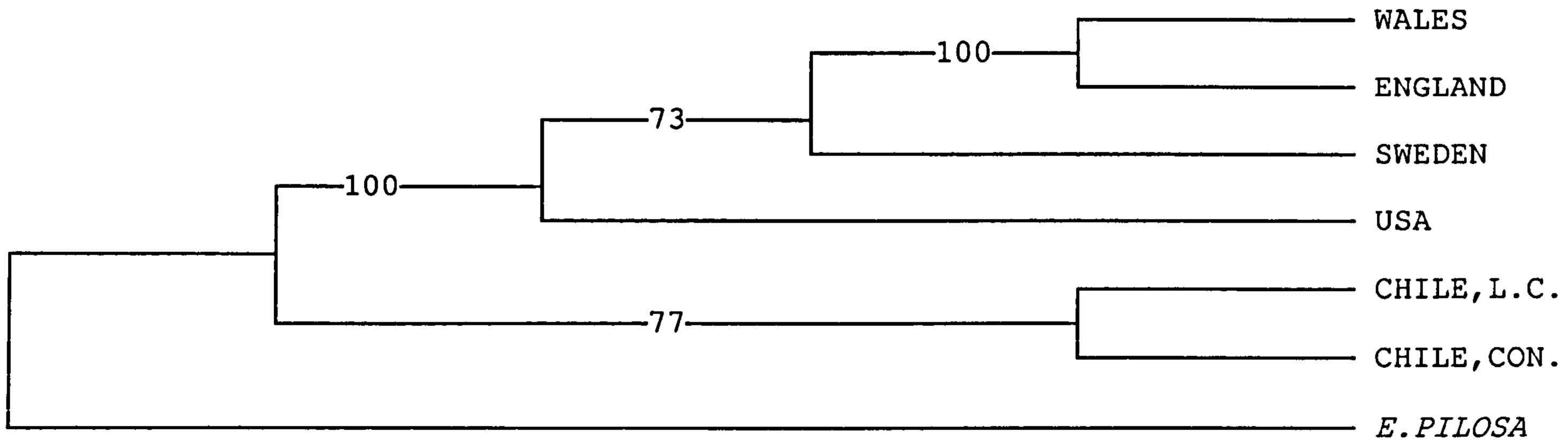


Figure 6.18. Phylogenetic tree formed using maximum parsimony, the heuristic search used which gave a single tree with a length of 217 steps.
Bootstrap values associated with branches were derived from 100 replicates.

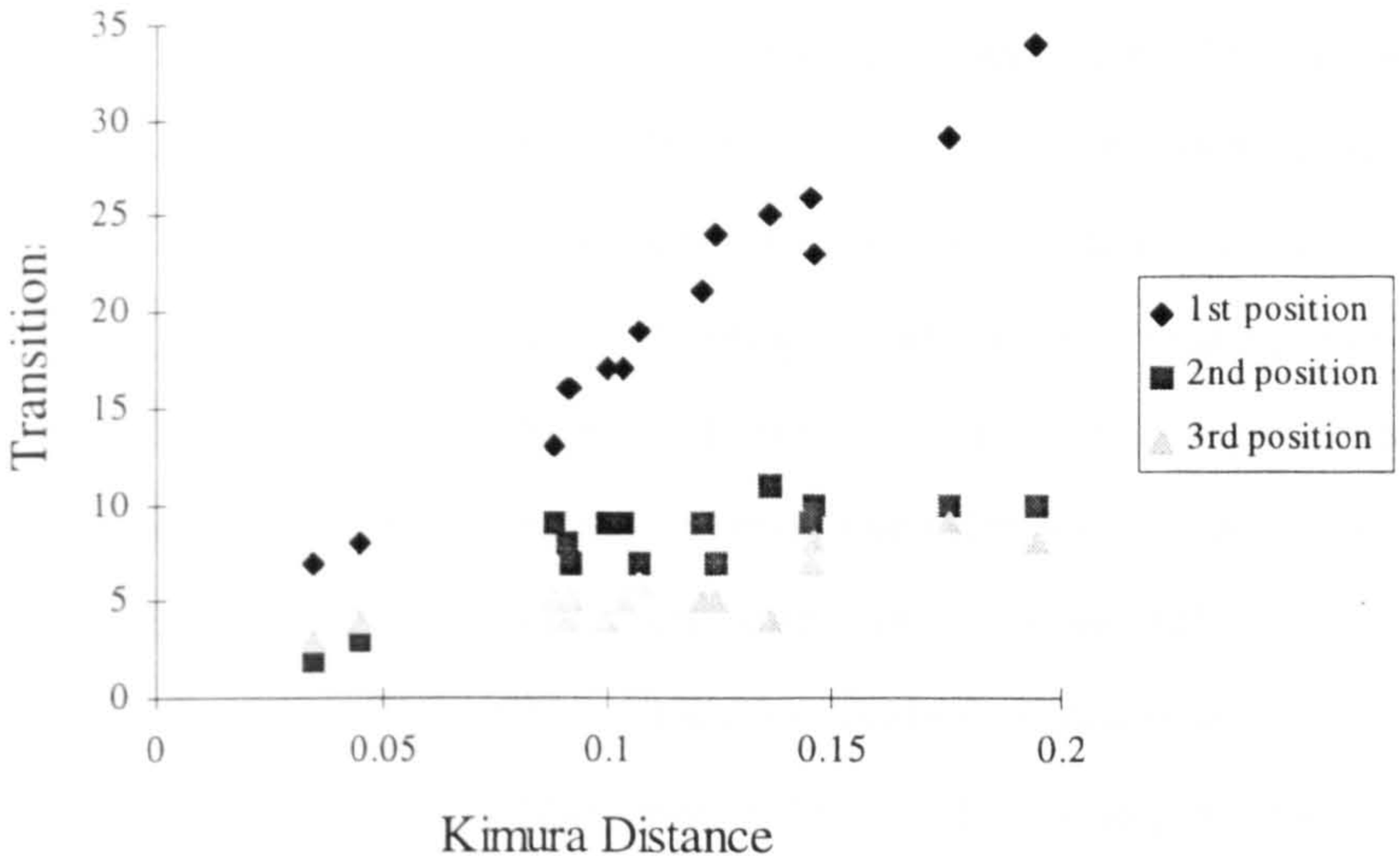


Figure 6.14. Number of transitions at each codon position plotted against the Kimura two-parameter distance for COI sequences of *C. hyalina* samples from locations around the Atlantic basin and the South Eastern Pacific.

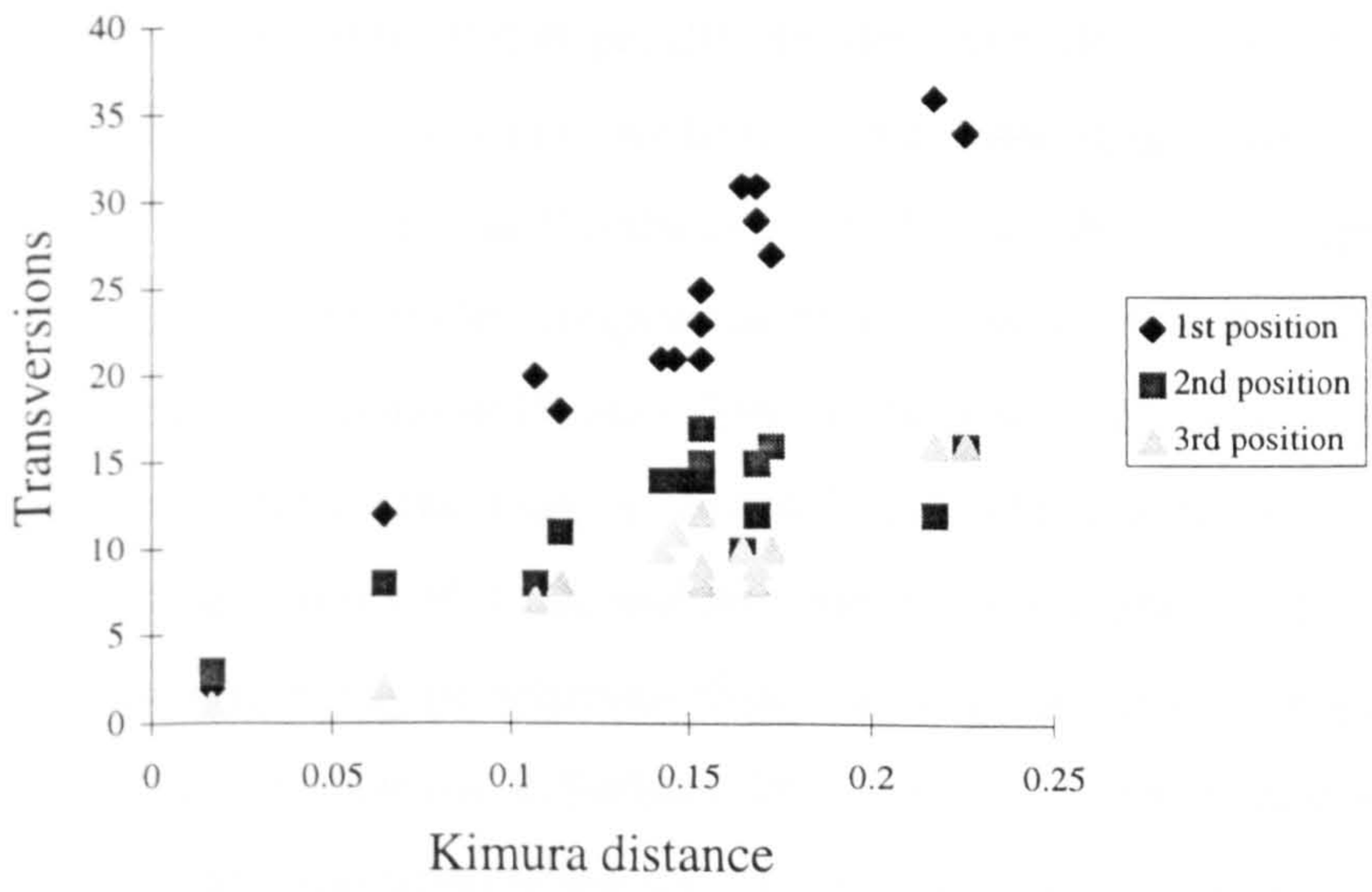


Figure 6.15. Number of transversions at each codon position plotted against the Kimura two-parameter distance for COI sequences of *C. hyalina* samples from locations around the Atlantic basin and the South Eastern Pacific.

6.4. DISCUSSION

Considerable differences are apparent between samples of *C. hyalina* from Chile and Wales. The numbers of autozooids produced by Chilean and Welsh colonies under identical conditions in the cross-fertilisation experiment were significantly different. The Chilean colonies were considerably smaller than the Welsh colonies, suggesting that Chilean colonies either grow at a slower rate naturally or found the experimental conditions sub-optimal. Welsh *C. hyalina* are predominantly found on the relatively ephemeral fronds of macroalgae (Hayward & Ryland, 1979; Cancino, 1986) whereas, Chilean colonies are found on the more stable substratum of kelp holdfasts (Cancino *et al.*, 1991). It seems likely that faster growth on a short-lived algal substratum would be heavily selected for. It is also possible that the culture regime implemented in the experiment may have hindered the Chilean colonies, but this further emphasises the considerable physiological differences between the two populations. Chilean colonies produced a low number of frontal males per autozooid in the cross treatments. However, much higher numbers were found in the controls when another Chilean colony was present. This may suggest different life history strategies between the Chilean and the Welsh. In the absence of conspecific sperm, Chilean colonies appear to minimise reproductive output. This may allow an increase in overall size and thus, if conspecifics are detected later on, higher overall reproductive output is possible due to the greater investment in colony size (Hayward & Ryland, 1975; Jackson & Wertheimer, 1985). Welsh colonies appear to maintain high levels of frontal males irrespective of the presence of conspecifics, which was also observed by Hunter & Hughes (1993a). Numbers of frontal males are actually higher in cross treatments than in controls. In the absence of local conspecifics, by increasing the number of males and thus the sperm concentration, a greater fertilisation range may be achieved, thus reaching undetected compatible colonies (Pennington, 1985; Denny & Shibata, 1989). Even though basal males generally make up only a small proportion of the total number of male zooids, the omission of data on basal males makes observations on male sexual allocation somewhat tenuous (Hunter, 1991; Hunter & Hughes, 1993a). Nonetheless, it appears that the Chilean and Welsh colonies did not responding as if conspecifics were present in the cross treatments. This is exemplified by the total lack of female zooids produced by the Chilean colonies in the cross treatments. Cancino *et al.* (1991) found that Chilean colonies

grown in isolation produced no female zooids, whereas Welsh colonies grown in isolation produce both females and embryos (Hunter & Hughes, 1993a).

The RAPD profiling of the few progeny found to settle in the cross-treatment pots clearly shows that the progeny were produced by the Welsh colonies and that the Chilean colonies present did not sire the offspring. These results were also confirmed by microsatellite analysis (K. Hoare, unpublished data). It must be concluded that the progeny were either the result of selfing or contamination by exogenous sperm; the incidence of non-parental bands may perhaps suggest the latter. The occurrence of non-parental bands, however, is a problem frequently associated with the use of RAPDs in parentage analysis. The frequently cited study of Riedy *et al*, (1992) found 4.4 and 2.7 bands per generation for 5 primers in primates and humans, respectively. The rate of non-parental bands found in this study (0.04 bands per primer) is lower than that found by Riedy *et al*, (1992) but higher than in burying beetles (0.017)(Scott & Williams, 1993), lake trout (0.011) (Stott *et al.*, 1997) and teiid lizards (0.01)(Tirado *et al.*, 1997). Sperm contamination from outside the experimental treatment can not be ruled out. Self-fertilisation is a phenomenon which has been observed in *C. hyalina* (Hunter & Hughes, 1993a; McCartney, 1994), which could have led to the production of larvae. However, more recent studies have suggested that selfing does not occur in total isolation (P. Manríquez, pers. comm.). Some botanical evidence exists which suggests an external gamete source can increase the rate of selfing (Visser & Marcucci, 1984).Whether the larvae produced in the cross-fertilisation treatments are the result of selfing or allogamy cannot be categorically stated.

A major impediment to the interpretation of these results is the lack of larval settlement in the Chilean controls, despite the large numbers of embryos present. A possible explanation for this is that the Chilean control treatment colonies were siblings. The original 10 Chilean colonies were supplied growing on a shard of glass. Therefore it is possible that one colony released the majority of the larvae. Consequently, it is possible that the resulting ancestrulae placed together were, in fact, siblings. Recent experiments have shown that close siblings produce very limited numbers of viable offspring (P. Manríquez pers. comm.). It is also possible that the Chilean colonies were heavily stressed by the experimental conditions, resulting in abortion of the embryos. Under highly stressful conditions, Welsh colonies of

C. hyalina have been found to abort embryos (P. Manríquez pers. comm.). The production of high embryo numbers in the Chilean colonies does imply fertilisation had taken place. Chilean colonies produced male zooids in all the cross-fertilisation treatments, and it therefore seems probable that Chilean sperm was present, but was not successful in fertilising the Welsh colonies.

The topology of the phylogenetic trees obtained from population mitotypes indicates a strong correlation with geographic distance. Generally, there is a high degree of similarity in branching patterns between the various tree building methods, implying a robust result has been obtained. However, a discontinuity is observed in the branching pattern for the Chilean samples using the ML method, compared with that of the other two methods. This throws into question the relationship between the two Chilean samples, but it is clear that they both remain distant from the Atlantic basin samples. This observation is reinforced by the scanning electron micrographs and light microscope observations of Chilean colonies, which reveal a much higher number of frontal pores originating from lacunae than in British, Swedish or U.S. colonies.

The extremely high rate of sequence divergence within what has been previously thought of as a widely dispersed single species, suggests that at least two species may be involved. Percentage sequence divergence is at a level more commonly associated with intergeneric outgroups amongst invertebrates, for COI (Walton, *et al.*, 1997; Brunton, 1998; Juan *et al.*, 1998), although some interspecific marine invertebrate studies have yielded COI sequence divergence levels of equal magnitude (Brasher *et al.*, 1992; Ovenden, *et al.*, 1997; Ó Foighil *et al.*, 1998). The level of divergence between Wales and Plymouth populations is at a more typical level for intraspecific comparisons, but is still higher than might be expected. This suggests that these populations have been isolated for a considerable time, which might be expected in a species with such limited dispersal (Chapter 5). The two Chilean specimens also show high levels of sequence divergence (10 %). The levels of divergence observed are higher than reported in most intraspecific studies. Specimens collected from Las Cruces were found on *Macrocystis integrifolia* holdfasts whereas those from Concepción were found exclusively on rock surfaces. Recent observations between colonies from both substrata found in sympatry have indicated that those living on holdfasts reproduce much earlier than those on rocks (J. Cancino, pers. comm.). Such life-history differences might suggest that considerable differentiation has occurred

between the two populations living on the different substrata. Further investigation into morphological and life history traits may provide sufficient evidence to assign separate species status. Chile boasts at least 18 hippothoan species and is an area of prolific speciation for this group (Moyano, 1986). It appears that on the grounds of genetic differences all of the samples examined, other than those from Wales and England, should at the least be thought of as sibling species. Closer examination of morphological and life history traits seems advisable for all the populations examined. For example, McCartney working with the Maine population of *C. hyalina* makes no mention of presence of basal male zooids, despite male sexual allocation being a integral part of his studies (McCartney, 1994, 1997; Yund & McCartney 1994). However, recent communications have revealed that the apparent lack of basal males in the Maine population may have been an oversight by the author. Regrettably, the condition of the samples sent from the Maine population of *C. hyalina* prevented the precise identification of the basal zooid types.

A striking degree of genetic divergence has been found in a number of sibling species of marine invertebrates (Palumbi & Benzie, 1991; Solé-Cava *et al.*, 1991; Rogers, *et al.*, 1995; Manchenko & Radashevsky, 1998). A possible explanation for this observation is that the rate of morphological evolution is much slower than the rate of molecular evolution. Selection may be acting to stabilise morphological traits, while molecular differences are steadily accumulated over time (Palumbi & Benzie, 1991). The identification of bryozoan species by skeletal morphology alone has been subject to scepticism (Levinton *et al.*, 1991). Cryptic or sibling species are thought to be highly prevalent among marine taxa (reviewed in Knowlton, 1993). In general, the taxonomic integrity of cosmopolitan marine invertebrates is increasingly being brought into question, largely due to the identification of cryptic species using molecular systematics (reviewed in, Thorpe & Solé-Cava, 1994). Greater scrutiny of the cosmopolitan "*C. hyalina*" population may well yield morphological or life history differences.

In conclusion, it can tentatively be suggested that the Chilean colonies of *C. hyalina* have not only evolved reproductive isolation mechanisms from Welsh colonies, but also exhibit a degree of morphological differentiation, which may be sufficient to clearly assign separate species status. Certainly, an in-depth morphometric analysis and a more conclusive demonstration of reproductive isolation

in sympatry would be likely to show this to be the case. Sequence data indicate very high levels of divergence between the majority of the sites examined. Morphological differentiation has either been strongly selected against or remains, as yet, undetected. In the light of this studies observations of variation in pore chamber number the later option appears to be the most likely. Evidently, extreme care must be taken when comparing seemingly widely dispersed marine species. Further studies of this nature are likely to yield a multitude of cryptic invertebrate species .

CHAPTER 7.

GENERAL DISCUSSION

7.1. Genotype-environment interaction

In this study, laboratory tests provided little evidence of genotype-environment interaction in either colony astogeny or sexual allocation for *C. hyalina* or *E. pilosa.* Previous studies using *C. hyalina* have found genotype-environment interaction in the production of sexual zooids amongst some clones under different temperature and food regimes (Hunter & Hughes, 1995). Significant levels of genotype-environment interaction have also been observed in *E. pilosa* through the production of different colony forms, growth rates and polypide life spans when subject to varying algal concentrations (Bayer *et al.*, 1994). Evidence of genotype-environment interaction provides support for the Tangled Bank Hypothesis for the maintenance of sexual reproduction (Bell, 1982). It is hypothesised that the release of sexually produced offspring with diverse genotypes, into a heterogeneous environment, should reduce competition between siblings that would normally occur between asexually produced progeny. Care must be taken in the interpretation of the results from the present study due to the limited number of genotypes used to identify inter-clonal variation. The lack of genotype-environment interaction may also be unsurprising in terms of chronic toxicant stress as responses to such stress are thought to have a complex genetic basis, resulting in a wide but relatively uniform phenotypic response (Hoffmann & Parsons, 1991).

7.2. Colony form and sexual allocation

Despite the lack of observable genotype-environment interaction, large variations were visible between genotypes in both sexual and morphological parameters, confirming the findings of other authors (Hughes, 1989; Hunter & Hughes, 1993b). A number of general trends were detected in the results. Firstly, sexual investment increased in relation to the number of feeding zooids, probably because of increased efficiency of food capture, allowing energetic allocation into

sexual processes. Colony form altered under stressful conditions. Under optimal conditions with rapid colony proliferation, *E. pilosa* colonies develop into stellate forms (Okamura, 1992; Bayer & Todd, 1996; Chapters 2 & 4). Subjected to moderate stress, however, the colonies grew more circular. At the other end of the scale, in nutritionally impoverished conditions *E. pilosa* colonies form uniserial runners, which may enable the location of more suitable micro-environments (Jebram & Rummert, 1978; Chapter 3). In contrast to *E. pilosa*, *C. hyalina* colonies form more circular colonies in conditions of rapid growth and become more lobate in stressful environments. Highly stressful environments also result in the formation of large numbers of basal male zooids in *C. hyalina* (Chapters 2 & 3). Such a switch to sexual production at the expense of autozooids represents a 'trade off' between somatic and sexual function (Stearns, 1989). This shift in production may well increase the likelihood of the perpetuation of the parental genes, by producing large numbers of relatively physiologically inexpensive gametes prior to the demise of the colony.

7.3. Variation between *E. pilosa* colonies from Church Island and Hell's Mouth

The examination of differences between *E. pilosa* genotypes from two contrasting sites yielded very pronounced variation with respect to colony growth form and sexual production (Chapters 2,3 & 4). *E. pilosa* astogeny appears to be predominantly genetically predetermined, although relative growth rate appears to have some influence since, as discussed above, faster growing colonies become more stellate (Okamura, 1992; Bayer & Todd, 1996; Chapters 2 & 4). The increase in relative perimeter may alleviate problems of restricted growth associated with circular form. The iterative colony perimeter progressively reduces relative to the increasing colony area over time (Hughes & Hughes, 1986a). Advantages may also be derived from increasing the peripheral zone for escape of excurrent flows from assemblages of feeding lophophores (Grünbaum, 1995). Colonies exhibiting the most stellate form were also those which occurred in conditions where 'long' median proximal spines were formed and the number of opesial spines was comparatively high (Chapter 4; Bayer *et al.*, 1997). The formation of surface spines is thought to increase resistance to excurrent flows, which subsequently reduce colony feeding efficiency (Grünbaum, 1997). Stellate form may allow feeding efficiency to be maintained while reaping the benefits of spine formation in protection against

abrasion and an increased boundary layer above the colony allowing lophophore extension in high current velocities (Chapter 4). Circular colonies may be favoured in areas where spine formation is not a prerequisite for survival, but colony life-span is likely to be short due to intense competition. Circular shape may enable colony resources to be efficiently translocated to zooids involved in sexual reproduction, through increased zooidal connectance. Consequently, reproduction can take place rapidly, but at the cost of lowered fecundity due to reduced colonial size (Jackson & Wertheimer, 1985). However, circular growth form may not be an adaptation, but a consequence of slower growth rate, where in-filling rate is equal to the rate of growth of the primary axis (Okamura *et al.*, 1992).

The striking variation in sexual productivity observed between CI and HM colonies in Chapters 2 and 3, was resolved by the addition of *Isochrysis galbana* into the *Rhinomonas reticulata* diet (Chapter 4). It appears that the *I. galbana* provides a nutritional component necessary for sexual reproduction in the HM *E. pilosa* colonies. This strengthens concerns expressed by other authors regarding the utility of a mono diet for this species (Bayer *et al.*, 1994).

7.4. Phenotypic plasticity

The high level of plasticity observed in *E. pilosa* colonies is likely to be an adaptation to its largely passive planktonic dispersal and sedentary adult life. Conditions that adult colonies are liable to encounter will be highly variable (Strathmann, 1985). Phenotypic plasticity under these circumstances therefore is advantageous. The polyphenic plasticity of extended median proximal spine formation observed in reciprocal transplants appears to be an excellent example of the advantages conveyed by such adaptive traits (Gottard & Nylin, 1995). Native colonies grew larger and survived better than transplanted colonies (Chapter 4), colony growth being a good measure of fitness in bryozoans, as colony size is directly proportional to reproductive output (Hayward & Ryland, 1975; Jackson & Wertheimer, 1985).

Plasticity of tentacle number was found to be very restricted in *C. hyalina*, but variable in *E. pilosa* (Chapter 3). Presumably, no selective advantage is conveyed by the ability to vary feeding current velocity in *C. hyalina* due the more conservative growth habit of this species. The reduced degree of plasticity exhibited by *C. hyalina*

in relation to tentacle number and colony form, compared to *E. pilosa*, may be a reflection of the contrasting modes of larval dispersal exhibited by these species. The short distance dispersal exhibited by *C. hyalina*, would be likely to result in newly established colonies experiencing similar conditions to those their parents encountered. Consequently, the ability to display a high degree of phenotypic plasticity may be unnecessary.

7.5. Copper toxicity

Despite speculation that bryozoans may make good indicators of pollution (Scholz, 1990), it appears that both *C. hyalina* and *E. pilosa* show such high resistance to copper toxicity as to render them unsuitable for this role (Chapter 2). However, potential susceptibility to other types of pollution and the advantages conveyed by clonal replication may still mean that bryozoans are useful indicators of water quality. The hormesis phenomenon exhibited by colonies exposed to chronic copper contamination also suggests that investigation into the kinetics of growth control mechanisms could be undertaken using *C. hyalina* and *E. pilosa*.

7.6. Population genetics

Evidence obtained using RAPD analysis indicates that the demography of *C. hyalina* is highly structured over relatively limited spatial scales (Chapter 5). Such population structure is probably a consequence of extremely restricted larval dispersal, enabling the exploitation of localised areas of suitable habitat and increasing the chances of both fertilisation and colony fusion (Craig, 1994). Close proximity between siblings and parents, resulting from short range dispersal, may lead to philopatry which is thought to maintain genotypes best suited to the local environment (Shields, 1982). No structuring of populations was observed in *E. pilosa* samples over 10 km, which might be expected with respect to its planktonic mode of dispersal. However, considerable differences were detected between the CI and HM populations which are approximately 80 km apart (Chapter 5). This may be attributed to separation by prevailing marine currents. This finding may explain the large differences observed in performance between CI and HM clones in the laboratory experiments (Chapters 2, 3 & 4).

The short range dispersal of *C. hyalina* appears to have minimised gene flow between widely dispersed populations, enabling a high degree of genetic divergence to occur in isolation (Chapter 6). The degree of dispersal achieved by the larvae of sessile marine invertebrates is thought to have profound influences on the evolutionary fate of the organism (Jablonski & Lutz, 1983; Jablonski & Raup, 1995). The seemingly nominal morphological differentiation of this allegedly cosmopolitan species is probably due to high levels of stabilising selection maintaining this phenotype at an "adaptive peak" (Lande, 1985). Long-term morphological stasis is seen in many fossil bryozoans implying that stabilising selection has been maintained over geological time scales (Cheetham *et al.*, 1995). This suggests that cryptic speciation may be even more prolific than is already thought, not only within the Bryozoa but also among other marine invertebrates (Knowlton, 1993). Great care must be taken when interpreting results of studies on marine organisms ranging over large distances and possessing similar life history traits. The findings presented in Chapter 6 strongly suggest that the Chilean population of *C. hyalina* is a separate species to that found in British waters and should be classified as such.

APPENDICES

Appendix 1.1.

Formula for calculation of Relative Colony Perimeter (RCP) a dimensionless index of colony form (Jebram & Rummert, 1978; Bayer *et al.*, 1994).

$$RCP = (Perimeter^{2} / 4 \pi Area)$$

Appendix 2.1.

Example of a RAPD phenotype binary matrix for 80 individuals of *E. pilosa*. A total of 52 bands scored for presence (1) or absence (0).

1.1.1. 01000100100001100100000010010100100101110110010010010
1.1.1. 00001101100001000000011111011000100101100111010011001
1.1.1. 01100000100010000000100001100011011010100110100001000
1.1.1. 1110001011100000011001000010001100111110111010110010
1.1.1. 0110100010101100001001010011100100101100011010000011
1.1.1. 0110110010000100111000100011100100111001110100100 10
1.1.1. 1100101010000011111001010011000100101100011010011011
1.1.1. 0100001110101000001011010100001111111001110101000 00
1.2.1. 1010110010001010000001010111001100101000011011010011
1.2.1. 1100111010000010000011010111000100011000011010001011
1.2.1. 1100111010101010111100000000001100101010011010001010
1.2.1. 0010100010000010110001010001100100101000111011000010
1.2.1. 1001101010001000010011010110001100011000011010010010
1.2.1. 0010101011100000001001100010001100101100111011010011
1.2.1. 1000100010100010010011100001000100101100111010010010
1.2.1. 1100110011000010010010000111000100111010011010000010
1.1.2. 00000101100001100110010100000001100101100111010010000
1.1.2. 1010100110101111111010000111000100001110011010110011
1.1.2. 0100100010000010010011101000000100101010111010011010
1.1.2. 00001001100000000011001011010100100111100111011100011
1.1.2. 1010010010000010110011000101001100011010111010011010
1.1.2. 1000010010100000110001000011000101001100011010010010
1.1.2. 0100100010001110000010011111000100101110011010101011
1.1.2. 0110100010000010010010001010000100111010011011011000
1.2.2. 1101100010100011111011010010000100101100111010001011
1.2.2. 0010100010100010011000010000100100101010111010010010
1.2.2. 1010100010000011010011000101000100111100111010001010
1.2.2. 11011100100011110010111100000001110011000011010011010
1.2.2. 1100100011100010010011000101101100011000011010000011
1.2.2. 1000100010100101111011011010000100101100111010010000
1.2.2. 0010100010100110000001010101000101101110111010011010
1.2.2. 0010100010001010010011100101000100101100011011010010
1.1.3. 1010100010001010010011001010000100101000011010011011
1.1.3. 1101100010001011001001010110000100001000011010001011
1.1.3. 0100110010100010010001010010000100101010011010001010
1.1.3. 0110110010100011111110010110000100011010011010011010
1.1.3. 0000100010000100010001000100100100101110111010010001
1.1.3. 1110100111101100111001011010001101101100111010001011
1.1.3. 0000100010100100011001111000101100101010111011010011
1.1.3. 0110000010000001010001000101100100101010111010010010
1.2.3. 1011100011001001001001010100101100111000111010000010
1.2.3. 0000100010000000110001000100000100101010111010010011
1.2.3. 0010110011100100010001001011000100011100011010001010
1.2.3. 1110000010001000010001010010000100001100011010000010
1.2.3. 0000100010101011001001110110100100101010111010000000
1.2.3. 1010100010000000010011010011001100111000011010010011
1.2.3. 0110100010100011001000010100001100101100111011011011
1.2.3. 1000100010000010011000000100000100101100111010001010
1.1.4. 1110100010000101001001100011000100101010011010010010
1.1.4. 1101100010000010000011000110001101001010111010010010
1.1.4. 0100110011100110000011010101101100101100111010001001
1.1.4. 00001000101000100100000000011100100111100011011010011
1.1.4. 1000110011101100110000001110000101101110011010011010
1.1.4. 1100100011100011100000000011000100111010111010010011
1.1.4. 1110000010000011000011010111000100011110111010010011
1.1.4. 1110100011000011110000000101100110101000111010010011
1.2.4. 1101110010000010100010010100001110011010001010010010
1.2.4. 00001000100000000010001001010001100001100111011011010
1.2.4. 11100000100000010000001100001000100101010011010011000

Appendix 2.1. (cont.)

RAPD phenotype binary matrix contined.

1.2.4. 1 1 0 0 1 1 0 0 1 1 0 0 0 0 0 0 1 0 0 1 0 0 0 0 0 0 1 0 0 0 1 0 0 0 0 1 0 1 0 1 1 1 0 1 0 0 0 1 0 0 1
1.2.4. 1 1 0 0 0 0 0 0 1 0 1 0 0 1 0 0 0 1 0 1 1 1 0 1 1 1 1 1 0 0 0 1 0 0 1 0 1 1 0 0 1 1 1 0 1 1 0 0 0 0 1 1
1.2.4. 0 0 0 1 1 0 0 0 1 0 0 0 1 0 1 0 0 0 0 1 0 0 0 0 0 1 1 0 0 0 0 1 0 0 0 0 1 0 0 0 0 1 0 0 0 0 0 1 0 1 0 0 0 1 0 1 0
1.2.4. 1 1 1 0 1 1 0 0 1 1 0 0 0 0 1 0 1 1 0 0 1 1 0 0 0 1 1 0 1 0 0 1 0 0 0 0 1 0 0 0 0 1 0 0 0 0 1 1 0 0 0 0 1 0 0 1 0
1.2.4. 0 0 1 0 1 0 0 0 1 0 1 0 0 1 0 0 0 1 0 0 0 0 0 1 1 0 1 0 0 0 1 1 1 0 0 0 1 0 1 0 0 1 1 0 1 0 0 1 1 0 1 0
2.1.5. 0 1 0 1 1 1 0 0 1 0 0 0 0 1 1 0 0 0 1 0 0 0 1 1 0 0 1 1 1 0 1 0 1 1 0 0 1 1 1 0 1 0 0 1 1 0 1 1
2.1.5. 0 1 1 0 1 0 0 0 1 1 0 0 0 0 1 0 0 1 0 0 0 1 0 0 0 0 1 0 0 0 0 1 0 0 0 0 1 1 0 0 1 1 1 0 1 0 0 0 0 0 1 0
2.1.5. 1 0 1 0 0 0 0 0 1 1 0 0 0 1 0 0 0 1 0 0 1 1 0 1 0 0 0 1 0 0 1 1 0 0 1 0 1 0 1 0 1 0 1 1 1 0 1 0 0 1 1 0 0 1
2.1.5. 1 0 1 0 1 0 0 0 1 0 1 0 1 0 0 0 0 1 0 0 0 1 1 0 0 1 0 0 1 1 0 1 1 0 1 1 0 1 0 1 0 1 0 1 1 1 0 1 0 0 0 0 0 1 1
2.1.5. 0 1 0 1 1 0 0 0 1 1 0 0 0 0 1 0 0 0 1 0 0 1 1 1 0 1 1 0 0 0 1 1 0 0 0 0 1 0 0 0 0 1 1 0 1 0 0 1 1 1 0 1 0
2.1.5. 0 1 0 0 1 0 0 0 1 1 0 0 0 0 1 0 0 0 0 0 1 0 1 0 1 0 1 0 1 1 0 0 0 1 0 0 1 0 1 0 1 0 1 0 1 1 0 1 0 1 0 0 0 1
2.1.5. 0 1 0 0 0 0 1 0 1 0 1 0 1 0 0 0 1 1 0 0 0 0 1 1 1 0 1 1 0 0 1 0 1 1 0 0 1 0 1 1 1 0 1 0 0 0 0 1 0
2.1.5. 0 0 0 0 0 1 1 0 1 0 0 0 0 0 1 1 0 1 0 0 0 1 0 0 1 0 1 0 0 0 0 1 0 0 1 0 1 0 1 0 0 0 0 1 1 0 1 0 0 1 1 0 1 0
3.1.6. 1 1 0 0 1 1 0 0 1 1 0 0 1 1 0 0 0 1 1 0 0 0 0 0 0 0 0 0 1 0 1 0 0 0 0 0 0 0 1 0 0 1 1 1 1 0 1 0 0 1 1 1 0
3.1.6. 1 0 0 0 1 0 0 0 1 1 0 1 0 0 1 0 0 1 0 0 0 0 0 1 0 1 1 0 0 1 0 0 0 0 0 0 0 0 1 0 0 1 1 1 1 0 1 0 0 0 1 0 0
3.1.6. 0 1 0 0 1 0 0 0 0 0 0 0 0 0 1 0 0 1 0 0 1 0 0 0 0 0 0 0 1 0 1 0 0 0 0 0 0 0 1 0 0 1 1 1 0 0 0 1 0 0 1 0
3.1.6. 1 1 0 0 0 0 0 0 1 0 0 1 0 0 0 0 0 1 1 0 1 0 1 1 1 0 1 1 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 1 1 1 1 0 0 0 0 1 0 1
3.1.6. 1 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 1 0 0 1 0 0 0 0 0 1 1 0 0 0 0 0 0 0 0 1 0 0 0 1 0 0 1 1 0 0 0 1 1 0 1 0
3.1.6. 0 1 0 0 1 0 0 0 1 0 0 0 0 1 0 0 1 0 0 1 0 0 1 0 1 1 0 0 0 0 0 1 1 0 0 1 1 0 1 0 0 0 1 1 0 0 0 1 1 1 0 0
3.1.6. 1 0 0 0 1 0 0 0 0 0 0 1 0 0 0 0 1 1 0 0 0 1 1 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 1 1 0 0 0 0 0 1 0 1
3.1.6. 0 1 0 0 1 1 0 0 0 0 1 1 1 0 0 0 1 0 0 1 0 1 1 0 1 1 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 1 1 1 0 0 0 0 1 1 0 0

Appendix 2.2.

UPGMA genetic distance matrix for *E. pilosa*.

	1.1.1	1.2.1	1.1.2	1.2.2	1.1.3	1.2.3	1.1.4	1.2.4	2.1.5	3.1.6
1.1.1	0	1.69	1.37	1.65	1.44	1.46	1.59	1.76	1.53	3.08
1.2.1	1.69	0	1.47	1.35	1.49	1.25	1.45	1.34	1.45	2.81
1.1.2	1.37	1.47	0	1.31	1.22	1.44	1.46	1.27	1.6	2.8
1.2.2	1.65	1.35	1.31	0	1.26	1.13	1.39	1.53	1.56	2.93
1.1.3	1.44	1.49	1.22	1.26	0	1.04	1.43	1.33	1.39	2.79
1.2.3	1.46	1.25	1.44	1.13	1.04	0	1.48	1.45	1.27	2.81
1.1.4	1.59	1.45	1.46	1.39	1.43	1.48	0	1.42	1.48	2.99
1.2.4	1.76	1.34	1.27	1.53	1.33	1.45	1.42	0	1.44	2.44
2.1.5	1.53	1.45	1.6	1.56	1.39	1.27	1.48	1.44	0	2.96
3.1.6	3.08	2.81	2.8	2.93	2.79	2.81	2.99	2.44	2.96	0

Slatkin's linearized Fst genetic distance matrix for *E. pilosa*.

	1.1.1	1.2.1	1.1.2	1.2.2	1.1.3	1.2.3	1.1.4	1.2.4	2.1.5	3.1.6
1.1.1	0	0.00983	0	0.00804	0	0.00057	0.00553	0.0116	0.00231	0.09183
1.2.1	0.00983	0	0.00098	0	0.00285	0	0.00123	0	0.00056	0.07353
1.1.2	0	0.00098	0	0	0	0	0.00023	0	0.00509	0.07114
1.2.2	0.00804	0	0	0	0	0	0	0	0.00467	0.08317
1.1.3	0	0.00285	0	0	0	0	0.00006	0	0	0.07215
1.2.3	0.00057	0	0	0	0	0	0.00248	0.00055	0	0
1.1.4	0.00553	0.00123	0.00023	0	0.00006	0.00248	0	0	0.00143	0.08723
1.2.4	0.0116	0	0	0	0	0.00055	0	0	0	0
2.1.5	0.00231	0.00056	0.00509	0.00467	0	0	0.00143	0	0	0.08086
3.1.6	0.09183	0.07353	0.07114	0.08317	0.07215	0	0.08723	0	0.08086	0

Appendix 2.2. (cont.)

Geographical distance matrix for *E. pilosa*.

	1.1.1	1.2.1	1.1.2	1.2.2	1.1.3	1.2.3	1.1.4	1.2.4	2.1.5	3.1.6
1.1.1	0	0.5	1	1	10	10	100	100	10000	70000
1.2.1	0.5	0	1	1	10	10	100	100	10000	70000
1.1.2	1	1	0	0.5	9	9	99	99	9999	69999
1.2.2	1	1	0.5	0	9	9	99	99	9999	69999
1.1.3	10	10	9	9	0	0.5	90	90	9990	69990
1.2.3	10	10	9	9	0.5	0	90	90	9990	69990
1.1.4	100	100	99	99	90	90	0	0.5	9900	69900
1.2.4	100	100	99	99	90	90	0.5	0	9900	69900
2.1.5	10000	10000	9999	9999	9990	9990	9900	9900	0	80000
3.1.6	70000	70000	69999	69999	69990	69990	69900	69900	80000	0

UPGMA distance matrix for *C. hyalina*

	1.1.1	1.2.1	1.1.2	1.2.2	1.1.3	1.2.3	1.1.4	1.2.4	2.1.5	2.2.5	3.3.6
1.1.1	0	2.25	1.98	2.11	2.37	2.33	3.25	2.65	3.84	3.93	6.7
1.2.1	2.25	0	2.7	1.72	2.63	2.58	3.33	2.65	4.19	4.24	6.67
1.1.2	1.98	2.7	0	2.39	2.54	2.55	3.59	2.92	3.94	4.03	6.82
1.2.2	2.11	1.72	2.39	0	2.71	2.48	3.34	2.68	4.1	3.99	6.89
1.1.3	2.37	2.63	2.54	2.71	0	2.63	3.16	2.85	3.73	4.32	6.57
1.2.3	2.33	2.58	2.55	2.48	2.63	0	3.38	2.51	3.33	3.98	6.92
1.1.4	3.25	3.33	3.59	3.34	3.16	3.38	0	2.84	3.46	3.77	6.4
1.2.4	2.65	2.65	2.92	2.68	2.85	2.51	2.84	0	3.8	4.02	6.66
2.1.5	3.84	4.19	3.94	4.1	3.73	3.33	3.46	3.8	0	3.25	7.04
2.2.5	3.93	4.24	4.03	3.99	4.32	3.98	3.77	4.02	3.25	0	7.19
3.3.6	6.7	6.67	6.82	6.89	6.57	6.92	6.4	6.66	7.04	7.19	0

Appendix 2.2. (cont.)

Slatkin's linearized Fst genetic distance matrix for *C. hyalina*.

	1.1.1	1.2.1	1.1.2	1.2.2	1.1.3	1.2.3	1.1.4	1.2.4	2.1.5	2.2.5	3.3.6
1.1.1	0	0.01869	0.01411	0.01689	0.02405	0.02362	0.05525	0.0343	0.09113	0.09603	0.28416
1.2.1	0.01869	0	0.02928	0.00541	0.02657	0.02516	0.0566	0.03535	0.10009	0.10292	0.27932
1.1.2	0.01411	0.02928	0	0.02254	0.02816	0.02976	0.06623	0.03854	0.09898	0.10424	0.29457
1.2.2	0.01689	0.00541	0.02254	0	0.03347	0.0273	0.06141	0.03511	0.10291	0.0977	0.30077
1.1.3	0.02405	0.02657	0.02816	0.03347	0	0.03409	0.04789	0.0385	0.08241	0.11276	0.26679
1.2.3	0.02362	0.02516	0.02976	0.0273	0.03409	0	0.06004	0.02602	0.06657	0.09832	0.30399
1.1.4	0.05525	0.0566	0.06623	0.06141	0.04789	0.06004	0	0.03592	0.06735	0.0829	0.24187
1.2.4	0.0343	0.03535	0.03854	0.03511	0.0385	0.02602	0.03592	0	0.08344	0.28253	0.28253
2.1.5	0.09113	0.10009	0.09898	0.10291	0.08241	0.06657	0.06735	0.08344	0	0.06519	0.3177
2.2.5	0.09603	0.10292	0.10424	0.0977	0.11276	0.09832	0.0829	0.09429	0.06519	0	0.33292
3.3.6	0.28416	0.27932	0.29457	0.30077	0.26679	0.30399	0.24187	0.28253	0.3177	0.09429	0

Geographic distance matrix for *C. hyalina*.

	1.1.1	1.2.1	1.1.2	1.2.2	1.1.3	1.2.3	1.1.4	1.2.4	2.1.5	2.2.5	3.3.6
1.1.1	0	0.5	1	1	10	10	100	100	10000	10000	13000000
1.2.1	0.5	0	1	1	10	10	100	100	10000	10000	13000000
1.1.2	1	1	0	0.5	9	9	99	99	9999	9999	12999999
1.2.2	1	1	0.5	0	9	9	99	99	9999	9999	12999999
1.1.3	10	10	9	9	0	0.5	90	90	9990	9990	12999990
1.2.3	10	10	9	9	0.5	0	90	90	9990	9990	12999990
1.1.4	100	100	99	99	90	90	0	0.5	9900	9900	12999900
1.2.4	100	100	99	99	90	90	0.5	0	9900	9900	12999900
2.1.5	10000	10000	9999	9999	9990	9990	9900	9900	0	0.5	12990000
2.2.5	10000	10000	9999	9999	9990	9990	9900	9900	0.5	0	12990000
3.3.6	13000000	13000000	12999999	12999999	12999990	12999990	12999900	12999900	12990000	12990000	0

Appendix 2.3.

Number of colonies of *Celleporella hyalina* were counted on the longest frond and its associated dichotomies of 5 randomly selected *Fucus serratus* plants (Wood & Seed, 1986). Collections were made from 10 locations along the Menai Strait between Beaumaris and Church Island (Fig. 2a & 2b).

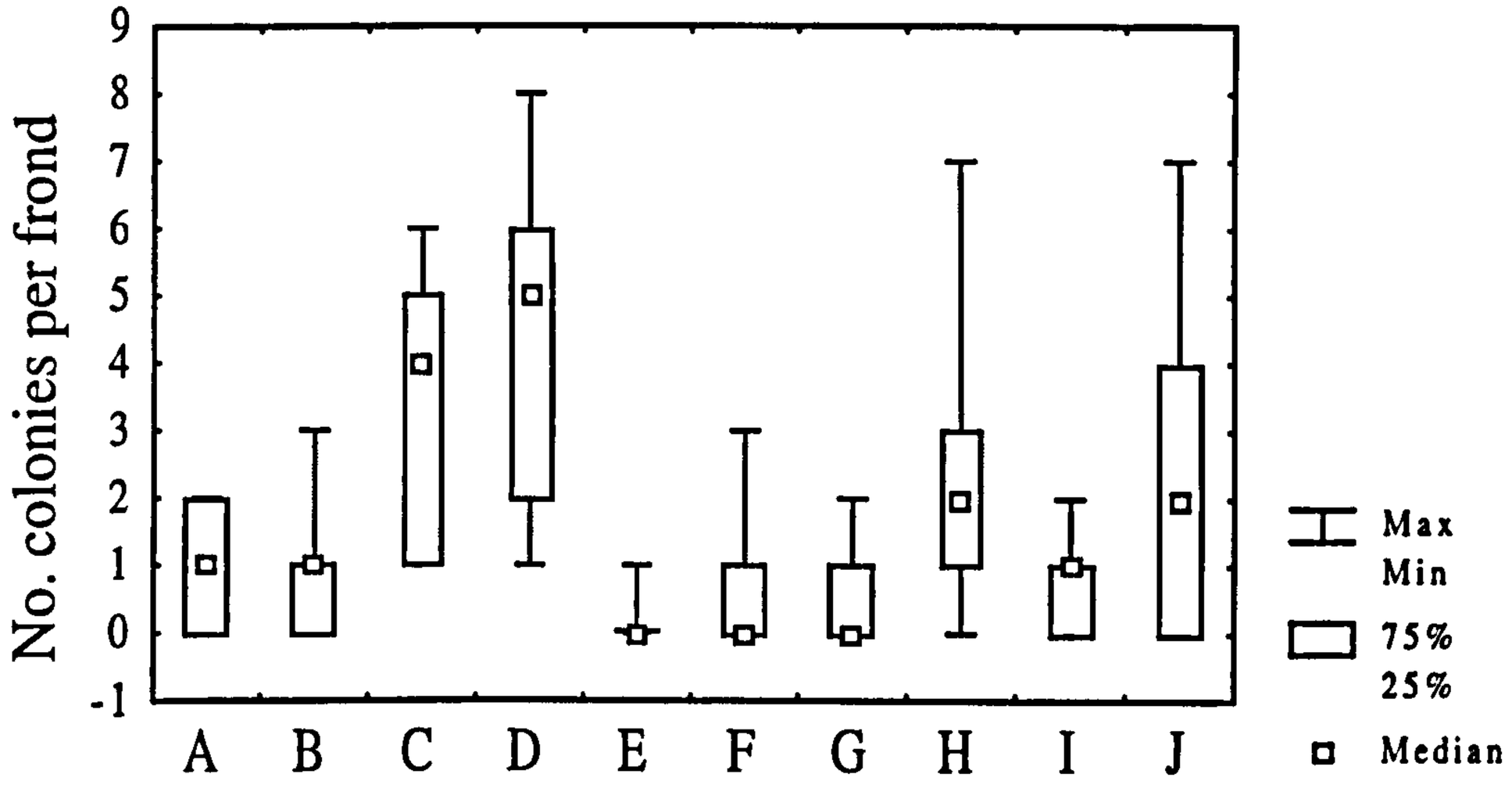


Figure 2a. Number of *C. hyalina* colonies found on fronds of *F. serratus* from 10 locations along the Menai Strait, North Wales; 75% to 25% boxes indicate upper and lower quartiles.

A- Gallows Point (NGR SH594749)

B- Derelict Garden (NGR SH590746)

C- Gazelle Jetty (NGR SH580740)

D- Glyn Garth (NGR SH576737)

E- Slipway (NGR SH572733)

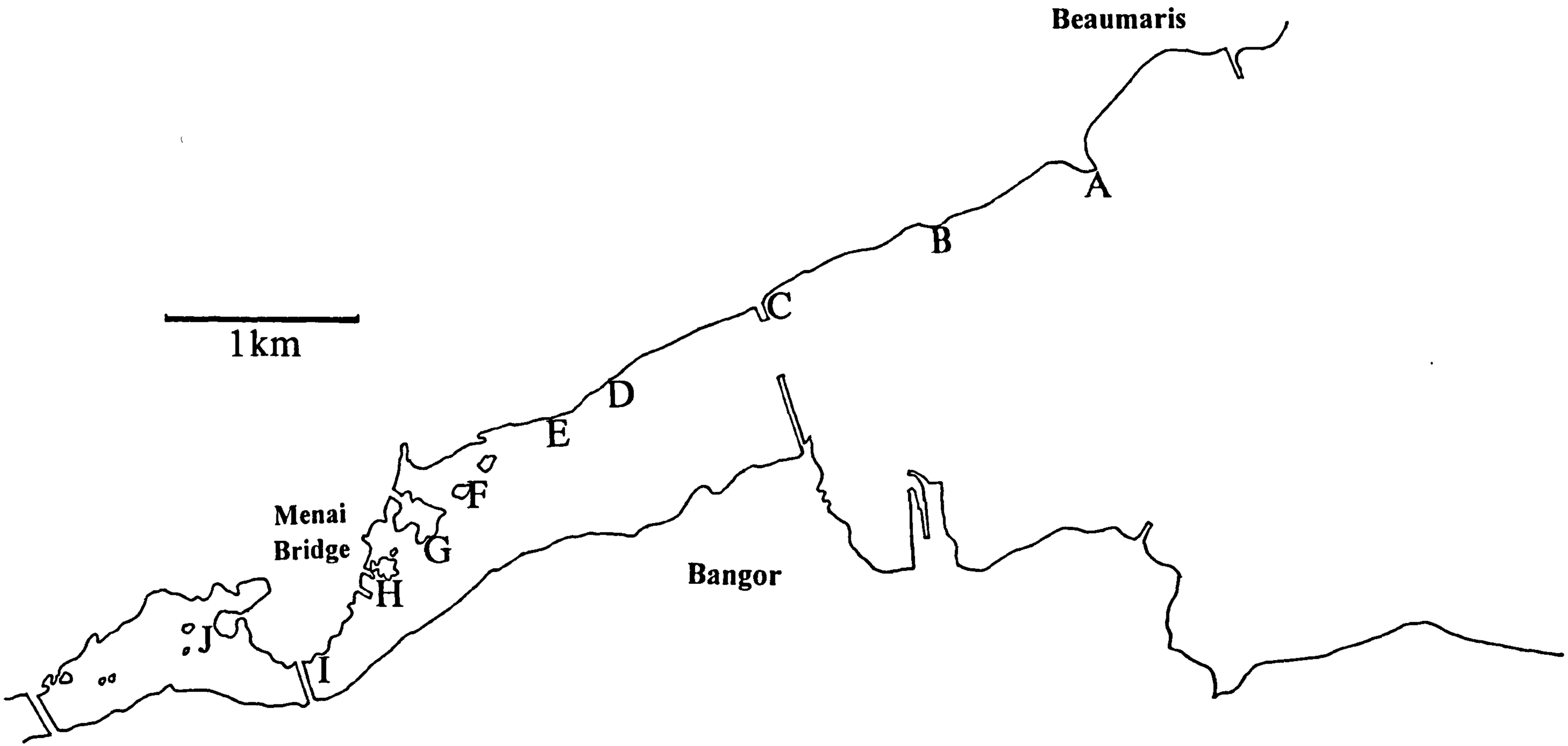
F- Ynys Castell (NGR SH568732)

G- Ynys Gaint (NGR SH564726)

H- Ynys Faelog (NGR SH561721)

I- Menai Bridge (NGR SH556715)

J- Church Island (NGR SH551717)



Appendix 2.3. (continued) **Figure 2b**. Site map of sampling locations along the Menai Strait. Five *F. serratus* plants were collected from each location to assess the occurrence of *C. hyalina* colonies.
A - Gallows point, B - Derelict garden, C - Gazelle jetty, D - Glyn Garth, E - Slipway, F - Ynys Castell, G - Ynys Gaint, H - Ynys Faelog, I - Menai Bridge, J - Church Island.

Appendix 3.1.

COI Sequence data obtained for *Celleporella hyalina* samples (365 bp).

Wales:
TGCATTAATTATGATTTTAAGTTCTTTTACAAGTGAGAGAGGTAGAGGTACTGGGTGAAC
AGTTTATCCACCCTTATCTTCTTCAATAGCCCATAGAGGGCCATCTGTTGATTTAACTATT
TTTTCTTTACATATGCTAGGAATTTCTTCTATTTTGGGTGCTATTAATTTTATTAGTACCAC
AGGCAATGCTCGTACTTCTTTGATAGGTATTATGCAACTACCTTTACTTATTAGTGCAACA
ACAATTACTGCTGTTTTGTTATTAATATCCTTACCAGTTTTAGCAGGTGCTATTACAATGC
TTTTGACAGATCGAAATTTGGATACTTCTTTTTTTGACCCATCTCGAGGGGGGACCCAAT

England:
TGCATTAATTATGATTTTAAGTTCTTTTACAAGTGAGAGAGGTAGAGGTACTGGGTGAAC
AGTTTATCCACCCTTATCTTTTTCAATAGCCCATAGAGGGCCATCTGTTGATTTAACTATT
TTTTTTTTACACATAGCAGGAATTTCTTCTATTTTGGGTGCTATTAATTTTATTAGTACCAC
AGGCAATGCTCGTAGTTCTTTGATAGGTATTATGCAAATACCTTTACTTATTAGTGCAACA
ACAATTACTGCTGTTTTGTTATTAATATCCTTACCAGTTTTGGCAGGTGCTATTACAATAT
TGTTAACAGATCGAAATTTAAATACTTCTTTTTTTGACCCATCAGGAGGAGGGACCCAAT

Sweden:
TGCACTAATTATGATAATTTATTCTTTCACAAGTAAGAGAGGTAGAGGTACCCGGTGAAC
ACTTAAACCCCCCCTTTGTTGTTCTATAAGCCATCCAGGCCCATCGGTAGAAATAACTATT
TTTTCTGTACACATATCGGGGGTTTCTTCTATTTTGGGTGCTATTAATTTTATTAGAACCA
CAGTTAATGTTCGTAGTTCCGGGATGGCCATAATGAAAATACCCCTATTTAGTAGTGCAA
CGATAATTACTGCTTTTTTATTATGGTGTTTTTTACCAGTTTTAGCCGGTGGTATTACCAT
GGTTTTTACCGATCGGAATTTAAATACTTCTTTTTTTGACCCCTCAGGTGGCGGGACCCAA
T

USA:
TGCGCTAATAATAATCCTGGGTGGTCTCACAAGTGAGAGAGGTAGAGGCACTGGGTGGA
CAATTAATCCTCCACTTTTTTTTTCTATAGCCCATCGGGGACCATCGGTTGATTTAACTAT
CTTTTTTTTACACCTAGCGGGGATTTCTTCTATTTTGGGTGCTATTAATTTTATTAGGACC
ACAGTTAATGTTTGCGGTTCCTGGATGGGCTTGATACAAATACCATTATTTATTAGGGCA
ACAAGGATTACCGCATCTTTTTTATGTTGTTAATTACCAGTTTTAGCGGATCCTATTACCA
TACTTTTGACAGATCGCAATTTAGATACTTCTTTTTTTTGACCCGTCTGGTGGGGGGACCCA
AT

Chile, Las Cruces:
GCCTTTAATAAAGATGGTGAGATTATTTACAATTGAGAGAGGGGGTGGAACAGGGTGGA
CAGTTAATCCTCCTCATTCTTTTTCAATTACCCATCGGGGACCGTCTGTTGATTTAACTAT
TTTTTCTGTACATATGGCAGGTATTTCTTCAATTTTGGGTGCGATTAATATTATTACTACT
ATTGTTAATGTTCGAGCTTCCGGGATGGGCTTGATGCAAATACCATTATTTATTTGGACTA
GGAGGATTACAGCATTTTTATTAATGTCGTCTTTGCCAGTTTTAGCTGGTGCTATTACGAT
GTTTTTGACAGATCGAAATTTCGATACGTCTTTTTTTGACCCATCTGGTGGTGGGACCCAA
T

Appendix 3.1. (cont.)

Chile, Concepción:
CCCTGTAATCAAGATGGTGAGGTCAATTACAATTGAGGGAGGGGGTGGAACAGGATGGA
CAGTTAACCTTCCTCCATCTTTTTCGATTACTTATAGAGGACCGTGTGTTGATTTAATTAT
TTTTTCTGTACATATGGCGGGGATTTCTTCAATTATGGGTGCCATTAATTTTATTACTACT
ATTGTTAATGTTCGAGCCTCCGGGATGGGGATGATGCAAATACCCCTATTTATTTGGACG
AGGAGGATTACAGCATTTTTATTAATTTCGTTTATGCCAGTTTTAGCTGGTGCTATTACGA
TGTTTTTGACAGATCGTCATTTGGATACGTCTTTTTTTGACCCATCTGGTGGAGGGACCCA
AT

Outgroup, *Electra pilosa* (Wales):
ACCCCTTCGTATTAACTTGCTCTTGTCAACTTTATTCATGGAAGGTGGCGCACCATCGAGA
GGATACACTCTGTATCCGCCCTTCTCAATACAGGGGGGTGCGTCTGTAGATTTTGTGATTT
TTTCTCTCCATCTGGCGGGGATGTCATCCATTATGGGGGCGATTAATATTATAACGACCAT
TTTTAACATGCGAAGTCCGGGGATAACTTTGATGAGAATACCATTATTTGTGTGGGCCAT
GTTGATCACAGCATATTTACTGATTGCGGTTATGCCGGTATTGGCTGGTGCAATTACGAT
ACTATTAACAGATCGTTATTTGGAACCAGCTTTTTTTGATCCTGCCGGTGGAGGAGACCCT
A

Appendix 3.2.

Distance: Kimura 2-parameter distance (transitions only).
No. of Codons in Subset: 121 of 121
Codon Position(s) Used: 1 2 3
Gap Sites and Missing Information Data: All such sites were removed from the subset data

OTU Labels
1.. WAL
2.. PLY
3.. SWE
4.. USA
5.. LCR
6.. CON

OTUs	1	2	3	4	5	6
1		0.0347	0.1037	0.1457	0.1081	0.1759
2			0.0919	0.1369	0.1246	0.1945
3				0.0918	0.1003	0.1217
4					0.0883	0.1462
5						0.0450
6						

Distance: Kimura 2-parameter distance (transversions only).
No. of Codons in Subset: 121 of 121
Codon Position(s) Used: 1 2 3
Gap Sites and Missing Information Data: All such sites were removed from the subset data

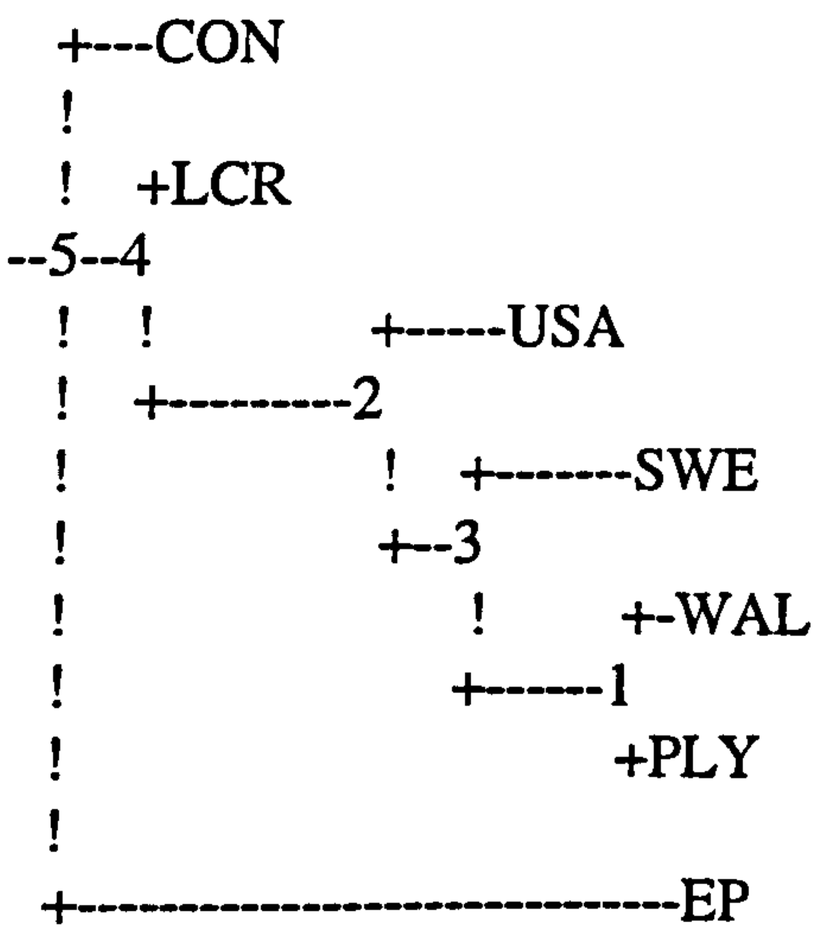
OTUs	1	2	3	4	5	6
1		0.0168	0.1536	0.1140	0.1688	0.1536
2			0.1461	0.1071	0.1688	0.1536
3				0.1425	0.2174	0.2260
4					0.1649	0.1727
5					0.0646	
6						

Appendix 3.2. (cont.)

PHYLIP output for maximum likelihood method.
Empirical Base Frequencies:

A	0.24518
C	0.17788
G	0.20740
T(U)	0.36954

Transition/transversion ratio = 2.000000
(Transition/transversion parameter = 1.630200)



remember: (although rooted by outgroup) this is an unrooted tree!
Ln Likelihood = -1865.80804
Examined 57 trees

Between		Length	Approx. Confidence Limits	
5	CON	0.06335	0.03039	0.09662
5	4	0.03112	0.00291	0.05925
4	LCR	0.02104	zero	0.04304
4	2	0.16490	0.11228	0.21880
2	USA	0.10222	0.06074	0.14424
2	3	0.03512	0.00444	0.06601
3	SWE	0.14104	0.09319	0.19015
3	1	0.11477	0.09319	0.15912
1	WAL	0.03096	0.01035	0.05171
1	PLY	0.02107	0.00315	0.03897

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