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Mate choice and reproductive investment in the cheilostome bryozoan Celleporella hyalina (L.).

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Mate Choice and Reproductive Investment in the Cheilostome Bryozoan Celleporella hyalina (L.)

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in candidature for the degree of Phylosophiae Doctor

by

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Summary of the Dissertation

In the present research several aspects of the reproductive biology of the marine hermaphroditic bryozoan, Celleporella hyalina (Linnaeus, 1767) were investigated. First (see Chapter 2), aliquots of different ages from a stock of allosperm suspension were used to fertilize a series of virgin ramets, so characterizing the decay in fertility of released sperm and any effects of sperm ageing on subsequent embryogenesis and larval metamorphosis. The effect of temperature on the above variables was also investigated. The fertile half-life of C. hyalina sperm was about 1-2 h, although significant decay in fertility occurred within a few minutes after release. Sperm ageing showed no deleterious effects on embryogenesis, larval viability, or metamorphosis. No clear effects of temperature on sperm ageing and fertilization success were found.

Allosperm storage was studied in colonies of C. hyalina (L.) under several experimental conditions (see Chapter 3). Recipient virgin colonies were exposed to sexually compatible allosperm suspension and the appearance of the last newly ovulated oocytes in the coelom was used to assess duration of sperm storage. The same experiments examined continuation of brooding cycle and brooding success throughout the period of allosperm storage. Similar observations were conducted on wild colonies of C. hyalina taken from the field and kept in reproductive isolation in the laboratory. Production of progeny in females zooids budded beyond the original colonial growing edge was taken as evidence of sperm movement. The results of the present study show that recipient colonies continue producing coelomic oocytes up to 5 weeks after exposure to allosperm suspension. Moreover, the progeny were produced not only by female zooids present at the moment of allosperm dosage but also by female zooids budded later, beyond the limit of the original growing edge. Since oocytes were not present in control colonies exposed to selfsperm, the results of the present study indicate that recipient colonies store sexually compatible allosperm and transport them within the colony in order to produce viable progeny.

The effect of water flow on both sperm release and fertilization success in colonies of C. hyalina (L.) was studied (see Chapter 4). Maximum numbers of released sperm were found at low and zero water velocities. Moreover, protruded male lophophores were observed only under those conditions. Fertilization success was studied in virgin colonies of C. hyalina (L.) exposed to compatible allosperm suspensions under different feeding activity and water flow conditions. Fertilization success was higher in colonies with more active feeding autozooids than in those with fewer feeding autozooids. High water flow conditions induced reduction in the proportion of protruded lophophores, and reduced the frequency of ovicells bearing progeny. Moreover, in all the experiments offspring were concentrated in areas of the colony bearing active feeding autozooids. The results of this study suggest that sperm release take place under similar conditions that enhance cross fertilization, with a possible role of feeding activity in bringing sperm to the proximity of receiver colonies.

Sperm competition and female choice was investigated in virgin colonies of C. hyalina (L.) exposed to sexually compatible allosperm cocktails (see Chapter 5). A microsatellite-based genotyping system was used to determine paternity. Progeny were mainly the product of outcrossing. In a few cases, a small proportion of progeny was attributed to self-fertilization. These results suggest that outcrossing is the main reproductive strategy in this species and that neither selective female choice nor sperm competition occur in C. hyalina.

Cryptic incompatibility allowing a flexible mating strategy to produce out-crossed progeny in the presence of allosperm and selfing when they were absent was not found.

The effects of mating sequence and temporal interval between matings (2 or 48 h) on sperm precedence in double-mated individuals were studied (see Chapter 6). Paternity was determined by using a microsatellite-based genotyping system. Settled colonies produced after short mating showed evidence of sperm mixing and first-male precedence. However, last colonies produced after both short and long mating intervals showed evidence of first-male precedence. When analyses were conducted using all the sampled progeny, low incidence of paternity by the second sperm donor (P_2) and absence of self fertilization were found. No effect of mating order on success of the second donor was found. Prevalence of outcrossing was also found. These results suggest that first-male precedence in C. hyalina may promote outcrossing under sperm limitation conditions, by acceptance of the first compatible allosperm to become available.

The effects of exposure to sperm suspensions and stressors on sexual allocation in colonies were studied (see Chapter 7). In the first experiment the effect of a waterborne factor on receptor colonies was studied. Adult colonies were exposed to compatible allosperm suspensions that had been filtered through 0.45 μm pores potentially able to remove cellular sized particles. As a control, receptor colonies were exposed to non-filtered allosperm suspensions. Appearance and growth of oocytes occurred only in the coelom of the control colonies. The active factor is not a dissolved molecule, but a particle that can be removed from water by filtration through 0.45 μm pores. This result plus the absence of developing oocytes in the receptor colonies exposed to similar concentration of selfsperm, suggest the operation of self/nonself recognition and an important and active role of allosperm in initiating colonial reproductive investment in C. hyalina. Prevention of colonial growth and others stressors were associated with production of basal male zooids. In other experiments, exposure to sperm suspensions of different degrees of genetic relatedness showed a virtual absence of production of progeny in those colonies exposed to closely related sperm (i.e. self and halfsibs). Finally, in experiments with sexually immature colonies, the onset of sexual reproduction was triggered by exposure to allosperm, resulting in the production of female zooids even before the appearance of male zooids.

Contacts between colonies of different genetic relatedness were studied under laboratory conditions (see Chapter 8). Moreover, observations were made on colonies growing on their natural substrata. Five types of responses were observed, from total fusion to overgrowth. Maximum degree of fusion, or morphological fusion, was manifested as morphologic interconnection between the adjoining colonies (i.e. production of coalescent zooids). Fusion occurred in all contacts between colonies of the same genotype, between parental colonies and their progeny and, between full and half sib colonies. In most cases the production of coalescent zooids was found. Absence of fusion occurred in all contacts between unrelated colonies and between some of the half sibs. Observations on wild colonies growing in contact with each other failed to reveal any incidences of coalescence. Non-aggressive overgrowth was confined to dead areas of one colony overgrown by zooids of the other healthy colony, independent of the genetic relatedness of the pairs. Differences in the fusibility between isocontact and allocontact suggest that colony specificity exists in C. hyalina, as has been found in other sessile colonial marine organisms.

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Chapter 1:
General Introduction.

Natural selection will favour maternal discrimination among potential offspring on the basis of their differing fitness, provided that correct assessments can be made before maternal resources have been irreversibly committed (Uyenoyama, 1993). One form of maternal choice is early discrimination, before fertilization, between male gametes from different sources. Whether or not female animals can influence the relative fertilization success of competing sperm within their reproductive tract has recently been tackled by evolutionary biologists (Eberhard, 1990; Eberhard, 1996; Birkhead, 1999; Clark et al., 1999).

Sexual selection by cryptic female choice can result from a female-controlled process or structure that selectively favours paternity by conspecific males with a particular trait over that of others who lack the trait when the female has copulated with both types (Eberhard, 1996). Sessile organisms which liberate sperm into the water, but retain eggs, generally will receive a mixture of gametes from various potential mates. Distribution by water currents may deliver gametes from different individuals, but conversely in hermaphrodites, self-sperm may return to its origin. Consequently, in species with indirect gamete transfer, some form of sperm capture and ability to discriminate among incoming sperm must be important and play a major role in their life history. One factor which may predictably influence the fitness of a potential offspring is the genetic relatedness of its parents. Both inbreeding and outbreeding depression have been documented in sessile organisms (Sabbadin, 1971; Price and Waser, 1979; Grosberg, 1987; Hunter and Hughes, 1993c; Cohen, 1996). Given a choice of male gametes, female discrimination on the basis of optimal parental relatedness would therefore be predicted (Waser and Price, 1993), and the evidence is reviewed by Birkhead (1998).

Gametic incompatibility, governing the outcome of mating opportunities, is known to occur in sessile groups (Scofield et al., 1982). Female discrimination between sperm on the basis of genetic similarity of conspecific males has been documented in lizards (Olsson et al. 1996 and 1999), insects (Wilson et al., 1997; Stockley, 1999) and in the compound ascidian Diplosoma listerianum (Bishop, 1996; Bishop et al., 1996).

In the present research. Celleporella hyalina (L.), an encrusting bryozoan commonly occurring on kelps and fucoids (Cancino, 1986), was used as the experimental organism. Sexually mature colonies are formed by polymorphic zooids: autozooids responsible for the filter feeding activity. androzooids or males zooids responsible for production of sperm. and gynozooids or female zooids responsible for the egg production (Plate 1). C. hyalina is a simultaneous hermaphrodite in which release of sperm into the water is believed to take place through the protruded male tentacles (Marcus, 1938), and fertilization takes place in retained eggs (Hughes, 1987). In gymnolaemate Bryozoans, sperm typically gain access through the coelomopore to partially-grown oocytes, retained in the ovary of maternal female zooid (reviewed by Ryland and Bishop, 1993). Mechanisms of sperm entry in C. hyalina are also discussed by Marcus (1938), DJ Hughes (1987) and Ostrovsky (1998).

Published evidence suggests that self-fertilization in Welsh C. hyalina is rare and associated with reduced fitness or abortion of embryos (Hunter and Hughes, 1993 c; Hoare et al., 1999). However, absence of progeny in Chilean colonies maintained under reproductive isolation has been reported (Cancino et al., 1991). Whether or not the progeny in C. hyalina are the result of self or cross fertilization, growing oocytes or early embryos are moved from the female coelom to special brood chambers called ovicells (Plate 1), and there the embryos remain for about 3-4 wk (Cancino and Hughes, 1988; Hughes, 1987). In C. hyalina, both the release of fully-developed lecithotrophic larvae and settlement can be induced under laboratory condition (Ryland, 1974). Larval release can be induced using a dark-light reaction, and then the lecithotrophic larvae (Plate 1) settle within 1.5-4.0 h (Cancino, 1988), producing the first postmetamorphic founding autozooid or schizoporelloid ancestrula (Cancino, 1988; Plate 1). Through non-sexual budding colony growth ensues with the eventual production of gymnozooids (i.e. female zooids) and androzooids (i.e. male zooids) would be produced (Plate 1).

Each autozooid bears a crown of tentacles, or lophophore, used to capture food particles (Plate 1) and has a U-shaped gut. In response to ageing and deleterious conditions, both structures can regress and be replaced by new ones (Gordon, 1977; Dyrinda, 1981 a and b). Thus, it is possible to distinguish active autozooids, i.e. those with a complete digestive

system and a functional feeding lophophore (Plate 1), and inactive or recycling zooids, i.e. those where the digestive system and lophophore have degenerated (Plate 1).

Colonies of C. hyalina can be cultured and propagated clonally in the laboratory by dividing colonies into independent pieces (ramets) on separates substrata (Hunter and Hughes, 1991). Consequently, the same genotype can be used simultaneously in different mating treatments. Thus clonal propagation, the rarity of self fertilization and production of progeny by controlled mating make C. hyalina a good experimental organism to investigate several problems that have never been investigated in the reproductive biology of this species. Moreover, the existence of published genetic microsatellite markers (Hoare et al., 1998) facilitate paternity analyses when several donor colonies are used as potential mates to fertilize receptor acting females in controlled matings.

In the following research I use an experimental approach to investigate unresolved aspects of the reproductive biology of C. hyalina, namely: the fertile life-span and the effect of sperm ageing on fertilization success (see Chapter 2); maximum duration of effective sperm storage, brooding time, allosperm storage and intra-colonial sperm movement (see Chapter 3); the effect of feeding activity and water flow on sperm release and fertilization success (see Chapter 4); the relative contribution of sperm competition and female choice (see Chapter 5); sperm precedence by sequential mating of ramets (see Chapter 6). Furthermore, to mimic nature, several kind of stressors relevant to adaptive selection (e.g. temperature, desiccation, mechanical damage and both physical and biological contact with the colonies) were used to assess their influences on sexual allocation (see Chapter 7). The possibility of self fertilization was investigated in most of the experiments. Finally, the consequences of genetic relatedness in the outcome of fusion between colonies were investigated (see Chapter 8).

Colonial invertebrates are ecologically important members of subtidal, hard-substratum communities, where they are major contributors to biodiversity (Jackson, 1977). Major questions of life history, such us those addressed above, are of fundamental interest for understanding processes generating genetic differences in natural populations, and so population divergence and speciation in the sea.

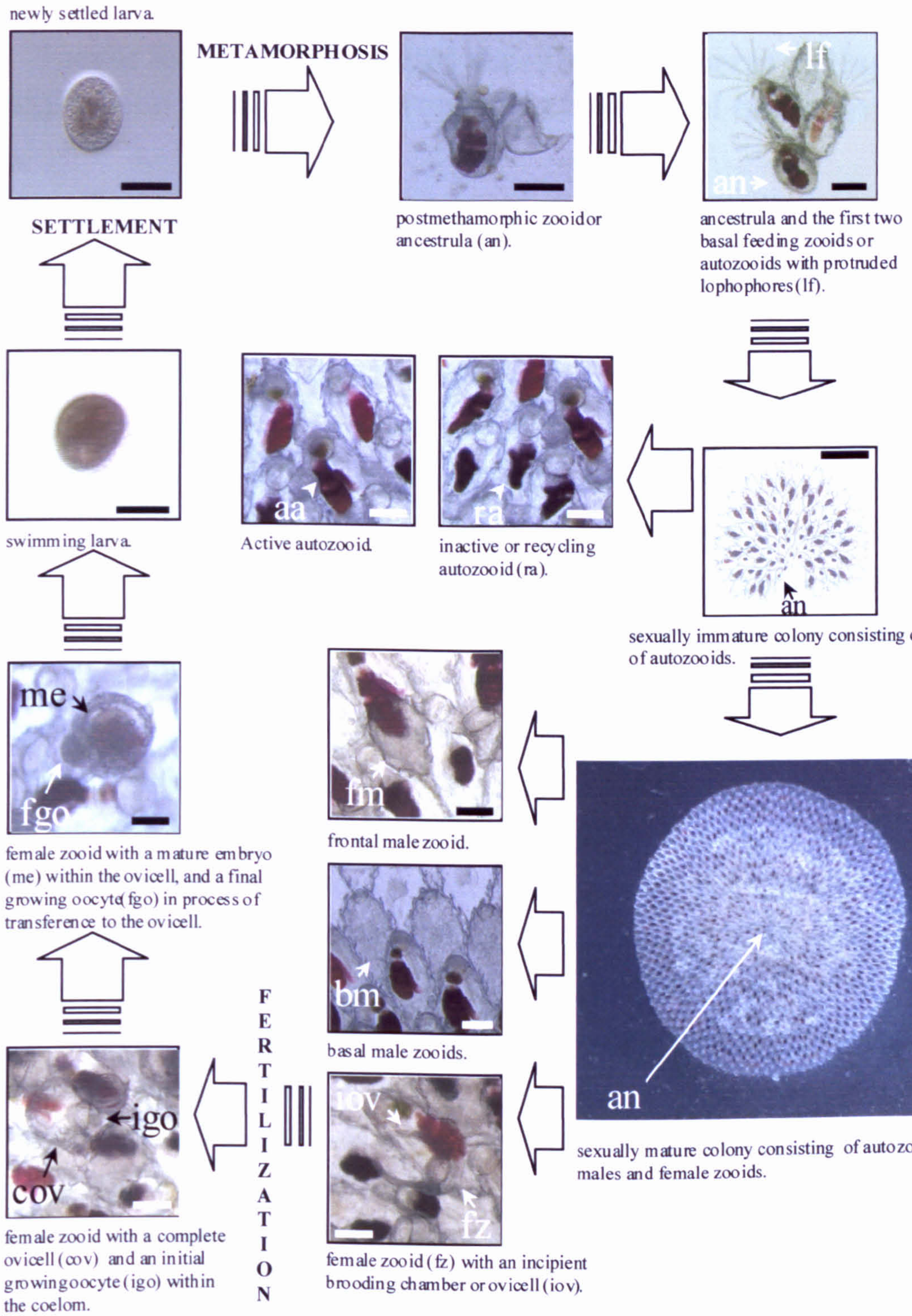

Plate 1: Celleporella hyalina life cycle and views of morphological details. In all the plates the scale bars are 100 µm, however in the sexually immature and sexually mature colony the scale bars are 1 mm.

Chapter 2:
Sperm fertile life-span and the effects of sperm ageing on fertilization success, embryogenesis and larval metamorphosis.

2.1 Introduction

Potential factors influencing fertilization success in marine invertebrates include attributes of the gametes, individual, population and environment (Levitan, 1991, 1993, 1995; Levitan et al., 1991, 1992; Yund, 2000). Among species that broadcast sperm, longevity of sperm after release should play an important role in reproductive ecology (Baccetti, 1979, Jamielson, 1986). Available information for marine invertebrates suggests that released gametes generally live only for a few hours, during which ageing reduces fertilization success (see Levitan 1995 for a review). Celleporella hyalina (Linnaeus, 1767) is a simultaneous hermaphrodite in which sperm, produced by male zooids, are broadcasted into the seawater, while embryos resulting from internal fertilization are brooded by morphologically specialized female zooids (Cancino and Hughes, 1988). Although self fertilization has been reported for C. hyalina (Yund and McCartney, 1994), other studies suggest that it is rare and produces abortive embryos (Hunter and Hughes, 1993c). Hence, it may be inferred that water-borne allosperm are required for successful fertilization and therefore that the fertile life span of sperm after release should play an important role in the reproductive ecology of C. hyalina, which may be taken as representative of any obligately outcrossing species that broadcasts sperm. An important advantage of C. hyalina as a model organism is that it can be cloned by fragmentation to produce genetically identical ramets (Hunter and Hughes, 1991; see Appendix 1). We predicted (1) that virgin ramets of C. hyalina in contact with fresh suspensions of compatible allosperm should produce more embryos than those in contact with older suspensions of the same allosperm, and (2) that ageing sperm should reduce success of embryogenesis, larval development and post-settlement metamorphosis.

2.2 Materials and Methods

2.2.1 Experimental clones and sexual compatibility

Six clones (A_1, D_1, E_1, J_1, M_1, Q_1) of C. hvalina were used in the present study. They were originated, propagated and reared using protocols described in Appendix 1. In a preliminary experiment, compatibility of potential sperm-donor and recipient clones was verified by the production of embryos and viable larvae. Viable larvae were considered those able to survive 3 wk after settlement. Virgin ramets of each recipient clone were exposed separately to allosperm suspensions released by the potential sperm donors. Ramets were exposed to 250 ml of unstirred allosperm suspension, with the addition of 0.5 ml of Rinhomonas reticulata cells as food, for 12 h. Under these conditions fertilization is more effective than in an aerated system lacking food (own unpublished data). As a control for self-fertilization, a ramet from each recipient clone was treated as above but using water lacking allosperm. As a further control, an extra ramet from each recipient clone was exposed to autosperm suspension for 2 h. In order to assess the reciprocal compatibility, the roles of sperm donor and recipient were reversed, using protocols equivalent to those above.

2.2.2 Sperm suspension and mating time

Having established the sexual compatibility of the experimental clones, sperm from the donor clones were obtained using standard protocols described in the Appendix 2. Receptor colonies were exposed to allosperm suspensions for 2 h using concentrations that were not limiting for fertilization (see Appendix 2).

2.2.3 Data collection and analysis

After being exposed to allosperm suspension, ramets were observed under a stereo microscope daily for 3-4 mo, by which time the last larva had been released. Ovulated coelomic oocytes (hereafter oocytes) and developing embryos (hereafter embryos) were counted using 400 x magnification and a 1200 x 1200 µm graticule placed beneath the substratum which colonies were growing. Ramets bearing embryos and releasing viable larvae were considered to have been successfully mated. Those not producing embryos and larvae were considered to have been unsuccessfully mated as a result of age-induced loss of sperm fertility.

Developing success was measured as the proportion of oocytes that developed into embryos. Metamorphosis success was measured as the proportion of embryos that, via larvae and ancestrulae, gave rise to young colonies surviving for at least 3 wk.

Because sperm donor clones were exposed to light for 15 min before sperm aliquots were collected, while preliminary counting of sperm took a further 10 min, the sum of these times was added to the experimental sperm-ageing times (above) before data analysis. The relationship between sperm age and number of developing zygotes was examined using linear regression analysis of semi-logarithmically transformed data. The sperm half-life ($SL_{0.5}$) was calculated as ln (0.5)/b, where b is the regression coefficient. Comparison between regression lines were made using analysis of covariance (ANCOVA)

2.2.4 Culture conditions

The experimental colonies were reared in 300 ml flat bottomed glass vessels (see Appendix 1) placed on a bench (Plate 1). The experiments were conducted in a controlled temperature room with a 12:12 dark/light photoperiod, the temperature being maintained at 16-17°C. Illumination was provided by two 40 W fluorescent tubes covered with a red diffuser. Larval release was induced through the dark / light reaction (Ryland, 1960), using a bench light and electronic timer to illuminate the experimental vessel from 06.00h to 08.00h daily. The colonies were fed every day using R. reticulata at concentrations of approximately 100 cells.μl^{-1}. Water changes were carried out every second day and the colonies and the acetate substratum cleaned once a week with the aid of a soft artist's brush. To obtain larval settlement, two acetate sheets, pre-conditioned by steeping in seawater to develop a microbial film (Mihm et al., 1981; Brancato and Woollacott, 1982; Keough and Raimondi, 1995, 1996), were placed in each experimental vessel, one covering the bottom and the other wrapped round the inner wall. Once mature embryos had became a larva, i.e. when their surface bore a ciliated corona (DJ Hughes, 1987), the settlement units were observed daily to assess the presence of newly settled colonies. To avoid losing swimming larvae, water changes were made 6 h after triggering larval release by which time most larvae able to produce a viable ancestrula would have settled (Orellana and Cancino, 1991; personal observations). Moreover, before its disposal the old water was passed through a sieve of

80 µm-mesh plankton netting. Larvae retained in the sieve were returned to the vessels along with the paternal colony and the settlement units.

2.2.5 Experiment 1: fertile life span

Once compatible clones had been chosen and minimum mating time ascertained, the fertile life span of released sperm could be examined experimentally. Recipient ramets were acclimatised to experimental conditions by maintaining them individually in a 300 ml vessel for at least 1 wk before exposure to allosperm suspension. As fertilization success in C. hyalina is positively correlated to food availability, colony size and proportion of feeding zooids that are active within the colony (own unpublished data), special care was taken to use ramets of similar in size and in number of active zooids.

The experiment was run four times, using the following pairs of clones: donor M_1, recipient A_1; donor A_1, recipient E_1; donor J_1, recipient Q_1; donor Q_1, recipient D_1. In each run, 17 virgin recipient ramets were chosen randomly from stock culture, these being of similar size, circular shape, lacking embryos and with > 65% of feeding zooids in active state, i.e. a complete digestive system and a functional feeding lophophore. 250 ml aliquots of allosperm, representing different time intervals since spawning, were removed at predetermined time intervals as follows: 0 min, 30 min, 1 h, 1.5 h, 2h, 3h, 4h, 6h, 8h, 10h, 12 h, 24 h, 36 h, and 48 h. A sample of 30 ml was taken from each aliquot for sperm counting and the remaining 220 ml were placed in a clean 300 ml glass vessel together with a recipient ramet for 1 h (Experiment 1) or 2 h (Experiments 2, 3 and 4) and then rinsed with aged 0.2 µm UV-treated seawater (hereafter FSW) for 5 min. The recipient ramets were then placed in clean experimental vessels with FSW and maintained under standard culture conditions (see below). In each experiment three ramets of the receptor clones were kept isolated from allosperm, thus serving as controls for self-fertilization.

2.2.6 Experiment 2: effect of temperature on fertile life span

The experiment was designed to assess any effect of temperature on sperm fertile life span during the time that receptor colonies were in contact with allosperm suspension. The experiment was run in two parts. In the part 1, ramets of clone M_1 were used as allosperm donors and virgin ramets of clone A_1 as recipients. In the part 2, ramets of clone A_1 were

used as allosperm donors and virgin ramets of clone E_1 as recipients. In each run, fifteen sperm-donor ramets were assigned randomly to each of three beakers placed in water baths at 12°C. 16°C and 18°C. In preliminary studies no effects of these temperatures on proportion of actively feeding zooids were found. Therefore, differences on fertilization or metamorphosis success in the present study should be more related to the effect of temperature on sperm longevity than to active sperm capture by receptor colonies. Allosperm suspensions were obtained from these ramets using the dark-light reaction, counted and administered in serially ageing aliquots to recipient ramets, as above. Throughout this procedure, vessels containing the recipient ramets were placed in the appropriate water bath. After exposure to allosperm for 2 h, recipient ramets were removed and rinsed with aged FSW for 5 min, then placed in clean experimental vessels with FSW and maintained under standard culture conditions at 16°C. Three recipient ramets were not exposed to allosperm but were otherwise treated similarly, as controls for self-fertilization.

2.3 Results

2.3.1 Mating compatibility

All matings between sperm-donor and recipient clones, including the reciprocal matings, resulted in the production of oocytes, viable embryos and ancestrulae (Table 1). All the mating combinations were able to produce progeny independently of which clone was used as allosperm donor or receptor. On the other hand, no vitellogenic oocytes or embryos were recorded from ramets maintained in reproductive isolation or exposed to autosperm suspension (Table 1). Experiments on the fertile life span of sperm (below) therefore would not be confounded by problems of mating incompatibility or self-fertilization.

2.3.2 Experiment 1: fertile life span

All recipient ramets exposed to allosperm produced oocytes, embryos, viable larvae and ancestrulae, whereas control ramets did not. No oocytes or embryos were normally found in ramets exposed to allosperm older than 12 h (Fig. 1). An exceptional embryo was recorded in a ramet of D_1 exposed to 24 h-old allosperm suspension from clone Q_1 (Fig. 2d). Number of embryos produced per colony decayed exponentially as allosperm suspension aged (Fig. 1). ANCOVA showed significant differences among the regression coefficients ($F_{3,32}$= 6.82, P<0.001) and among the elevations ($F_{3,35}$= 8.71, P<0.001) for cumulative number of embryos on ln (sperm age) at the four experiments (Table 2). Values of sperm half-life derived from the transformed data of each experiment ranged from 1 to 2 h (Table 2).

Most of the embryos recorded in the colonies successfully originated a newly settled colony, with values of development success of over 0.8 (Fig. 2). In a few exceptions, low values of development success were found associated with low numbers of embryos (Fig. 2). High values of metamorphosis success of over 0.8 were recorded (Fig. 3). When low values were recorded they were associated with low number of embryos in the colonies.

2.3.3 Experiment 2: effect of temperature on fertile life span

All out-crossed matings were successful, but no control ramets showed evidence of self-fertilization. The cumulative number of embryos produced by colonies decreased logarithmically with age of allosperm suspension (Fig. 4). Two ramets produced a single embryo after exposure to 24 h-old allosperm suspension. (Fig. 4d). No embryos were observed in colonies exposed to 36 and 48 h-old allosperm. Fertile half-life ranged from 1.11-2.03 h in the Part 1 and from 1.07-1.66 h in the Part 2 at the three experimental temperatures. In the part 1, ANCOVA showed no significant differences among the regression coefficients ($F_{2,18}$= 0.43, P>0.05) and among the elevations ($F_{2,20}$= 0.05, P>0.05) for cumulative number of embryos on ln (sperm age) at the three experimental temperatures (Table 3). Similarly, in the Part 2, ANCOVA showed no significant differences among the regression coefficients ($F_{2,17}$= 0.86, P>0.05) and among the elevations ($F_{2,19}$= 2.53; P>0.05) for cumulative number of embryos on ln (sperm age) at the three experimental temperatures (Table 3). Values of metamorphosis success in this experiment were almost always over 0.8 (Fig. 5). However, in a few cases low values were found when the number embryos in the colonies were also low as a consequence of sperm ageing.

2.4 Discussion

2.4.1 Self-fertilization

Absence of visible oocytes, embryos and developing larvae in all our ramets maintained in reproductive isolation or exposed to autosperm suspension confirmed the absence of self-fertilization. Earlier studies report that self-fertilization is infrequent in Welsh populations of C. hyalina (Cancino, 1983) and produces abortive embryos (Hunter, 1991; Hunter and Hughes, 1993c). In a sharp contrast, Yund and McCartney (1994) claim that in a North American population of C. hyalina self fertilization increases to high levels with increasing isolation from conspecific colonies. They explain this trend in terms of relative concentrations of auto and allosperm in ambient seawater. However, Welsh and North American populations are genetically differentiated (Hoare et al., 1999) and thus possibly differ in tolerance to inbreeding. Differing from studies on ascidians, where depression originated from selfing are expressed in the first generation (Cohen, 1996), it seems that in C. hyalina absence of self-fertilization is the extreme level of depression originated from selfing.

2.4.2 Effects of sperm ageing on success of development and metamorphosis success

The present study has demonstrated that sperm ageing has little or no effect on success of developing and metamorphosis. Lowest proportions of oocytes becoming embryos were found with aged sperm. However, these values correspond to proportions of small number of embryos (Fig. 2, 3 and 5), and so to make conclusion from these trends seems to be invalid. On the other hand, lowest values of metamorphosis success were also associated with small initial number of oocytes. Therefore, conclusions about negative effects of sperm age on metamorphosis success are equivocal, and it seems that once oocyte growth has been triggered, there is no effect of the age of the sperm and the completion of the full brooding cycle from embryo to settling progeny. Massive degeneration of autozooids has deleterious effects on development and metamorphosis success (personal observation). However, from the beginning to the end of the experiments all the colonies bore 60-75 % actively feeding autozooids.

2.4.3 Sperm longevity

Since self fertilization was absent in the control ramets and both allosperm concentration and mating time were non-limiting, it is possible to conclude that the exponential decay in the cumulative number of embryos produced by colonies with increasing age of allosperm was due to the loss of sperm fertility. From analysis of the decay in production of embryos is possible to conclude that the half fertile longevity is about 1-2 h. Similar values of longevity at different mating temperatures (12, 16 and 18°C) suggest that C. hyalina sperm are able to fertilize receptor colonies at temperatures normally occurring in the Menai Strait (Cancino, 1983; Owrid and Ryland, 1993; Kawai, personal communication).

The only available information on sperm longevity in an organism with a similar reproductive biology, involving internal fertilization and broadcast sperm, came from a study of Diplosoma listerianum (Bishop, 1998). This study noted that fertilization occurs with 24 h old sperm with a half fertile life of about 8 h. These studies highlight the same order of magnitude as C. hyalina. This highlights the restricted sperm longevity in marine organisms with ent-aquasperm (sensu Rouse and Jamienson, 1987).

Most published information on gametic fertility in marine invertebrates concerns species with external fertilization, in which fertilization frequency drops rapidly with distance from spawning adults (Pennington, 1985; Yund 1990; Grosberg, 1991; Brazeau and Lasker, 1992; Benzie et al. 1994). No information is available on C. hyalina sperm densities in the field, but sperm suspensions must rapidly become diluted after release as consequence of dilution. The behaviour of the C. hyalina sperm after release is largely unknown, however personal observations indicated synchronous release of sperm clouds by the protruded male zooids within a colony under both stagnant and low flow regimes (see Chapter 4). Moreover, male lophophores have been observed, in the present study, bending toward and releasing sperm into the exhalant currents of adjacent feeding lophophores, possibly thereby aiding dispersal. Synchronous release of sperm during periods of low water flow and dense stands of C. hyalina may to some extent compensate for dilution and limited duration of sperm fertility.

Genetic differentiation over small scales could be possible in sessile marine invertebrates

because fertilization success is a strong function of proximity to another spawning individual (Levitan, 1991; Levitan el al., 1992). Limited duration of sperm and intolerance of self fertilization suggest that efficient mechanisms of sperm capture and storage should have evolved to ensure successful fertilization. This seems to be the case in C. hyalina where evidence of sperm storage (see Chapter 3) and effect of water flow on fertilization success has been found (see Chapter 4).

The capacity of the lecithotrophic C. hyalina larvae to successfully metamorphose is negatively affected with swimming period (Orellana et al., 1996). Although all not lecitotrophic larvae are limited by energy supply (Miller and Hadfield, 1990), in general they are constrained to a short period of dispersal in the plankton (Strathmann, 1985). Therefore, low success of larval transport over long distances coupled with data on sperm longevity obtained in the present study, suggest restriction of the natural population to areas beyond which dispersion via larvae or sperm cannot be effective. This is in agreement with studies showing genetic differences in natural population of Welsh C. hyalina at relatively small spatial scales (Goldson, 1998). Under these circumstances reproductive isolation and cryptic or sibling species can arise easily as consequence of genetic divergence. However, the importance of dispersion by colonies drifting attached on pieces of macroalge cannot be discounted as a potential mechanism of gene flow.

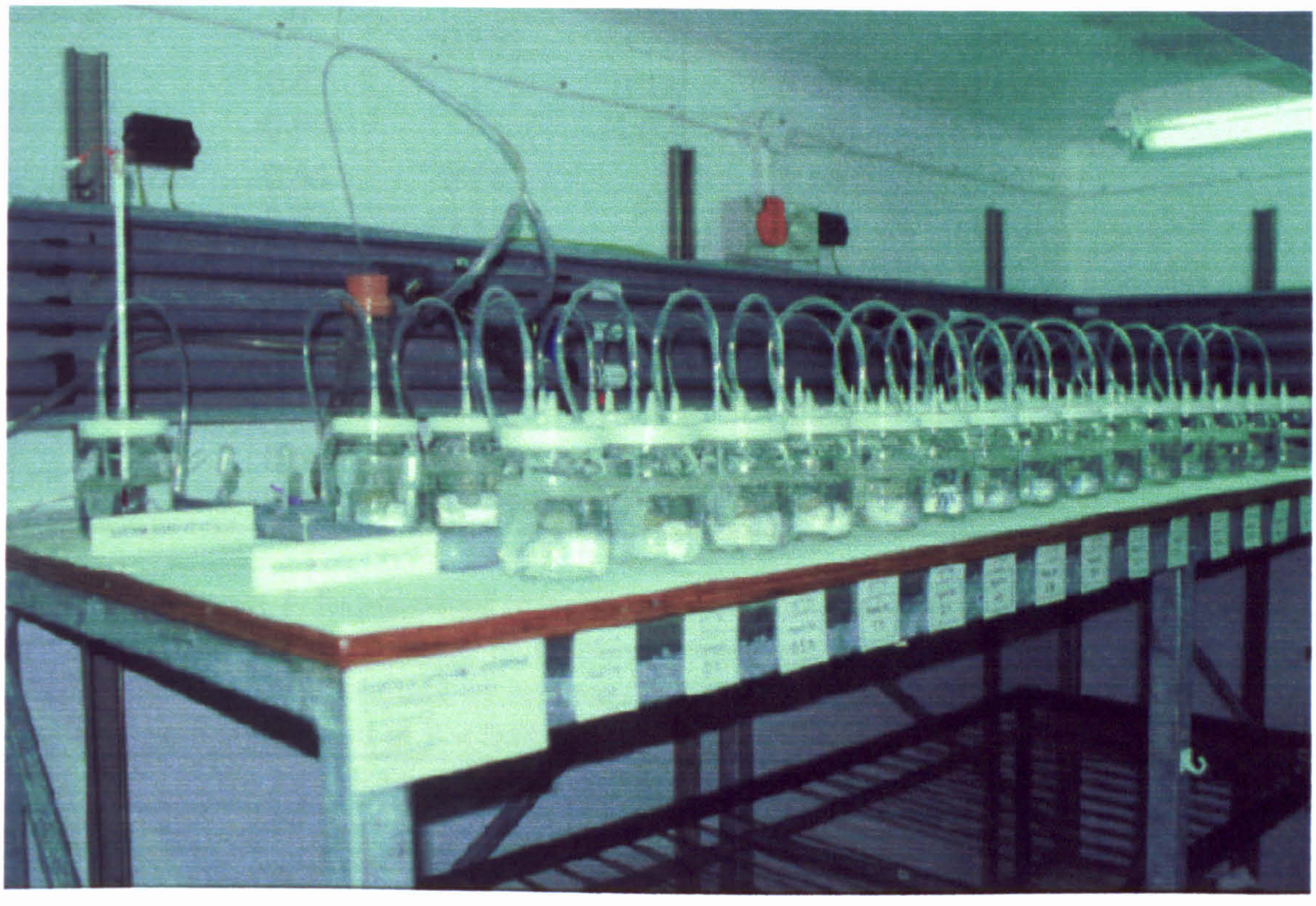

Plate 1: General view of the experimental set up for the sperm fertile life span in C. hyalina. In the front row flat bottomed vessels bearing colonies exposed to allosperm suspensions at 12 °C and in the back vessels containing colonies exposed at 18 °C.

Table 1. Mating compatibility between experimental clones of C. hyalina. Data are developing success and metamorphosis success (in parentheses), as defined in the text. Values above the diagonal represent mating with clones in the top row as sperm donors and clones in the first column as recipients. Values below the diagonal represent the reciprocal mating. Zeros in the first row of data represent lack of self-fertilization in reproductively isolated ramets, zeros in the diagonal represent the absence of self-fertilization in ramets exposed to autosperm suspension.

	Clone A_1	Clone D_1	Clone E_1	Clone J_1	Clone M_1	Clone Q_1
Reproductive Isolation	0	0	0	0	0	0
Clone A_1	0	0.63 (0.95)	0.59 (0.92)	0.43 (0.88)	0.53 (0.96)	0.39 (0.94)
Clone D_1	0.52 (0.90)	0	0.43 (1.00)	0.57 (0.88)	0.49 (0.94)	0.36 (0.89)
Clone E_1	0.55 (0.87)	0.64 (0.89)	0	0.49 (0.89)	0.52 (1.00)	0.62 (0.80)
Clone J_1	0.66 (0.96)	0.55 (1.00)	0.48 (0.98)	0	0.58 (0.94)	0.63 (0.97)
Clone M_1	0.55 (0.97)	0.48 (0.98)	0.51 (0.94)	0.60 (0.89)	0	0.63 (0.84)
Clone Q_1	0.66 (0.87)	0.50 (1.00)	0.67 (0.86)	0.64 (0.79)	0.54 (0.94)	0

Table 2. Experiment 1. Exponential regression coefficients relating cumulative number of embryos per colony of C. hyalina to ln transformed age of allosperm suspension. R^2 = proportion of variance explained by regression, $SL_{0.5}$ = fertile half life of allosperm. All regressions were significant at $P < 0.001$.

	Regression coefficient (h^{-1})	R^2	$SL_{0.5}$ (h)
Experiment 1:			
donor M_1, recipient A_1	- 48.82	0.68	1.64
donor A_1, recipient E_1	- 42.14	0.73	1.26
donor Q_1, recipient J_1	- 21.51	0.69	1.34
donor J_1. recipient D_1	- 59.99	0.81	1.99

Table 3. Experiment 2. Exponential regression coefficients relating cumulative number of embryos per colony of C. hyalina to ln transformed age of allosperm suspension at three different mating temperatures. R^2 = proportion of variance explained by regression, $SL_{0.5}$ = fertile half life of allosperm. All regressions were significant at $P < 0.001$.

Part 1: Donor M_1, recipient A_1	Regression coefficient (h^{-1})	R^2	$SL_{0.5}$ (h)
12°C	- 11.43	0.74	2.03
16°C	- 13.21	0.77	1.98
18°C	- 22.76	0.77	1.11
Part 2: Donor A_1, recipient E_1			
12°C	- 25.87	0.78	1.66
16°C	- 24.08	0.80	1.69
18°C	- 22.55	0.90	1.07

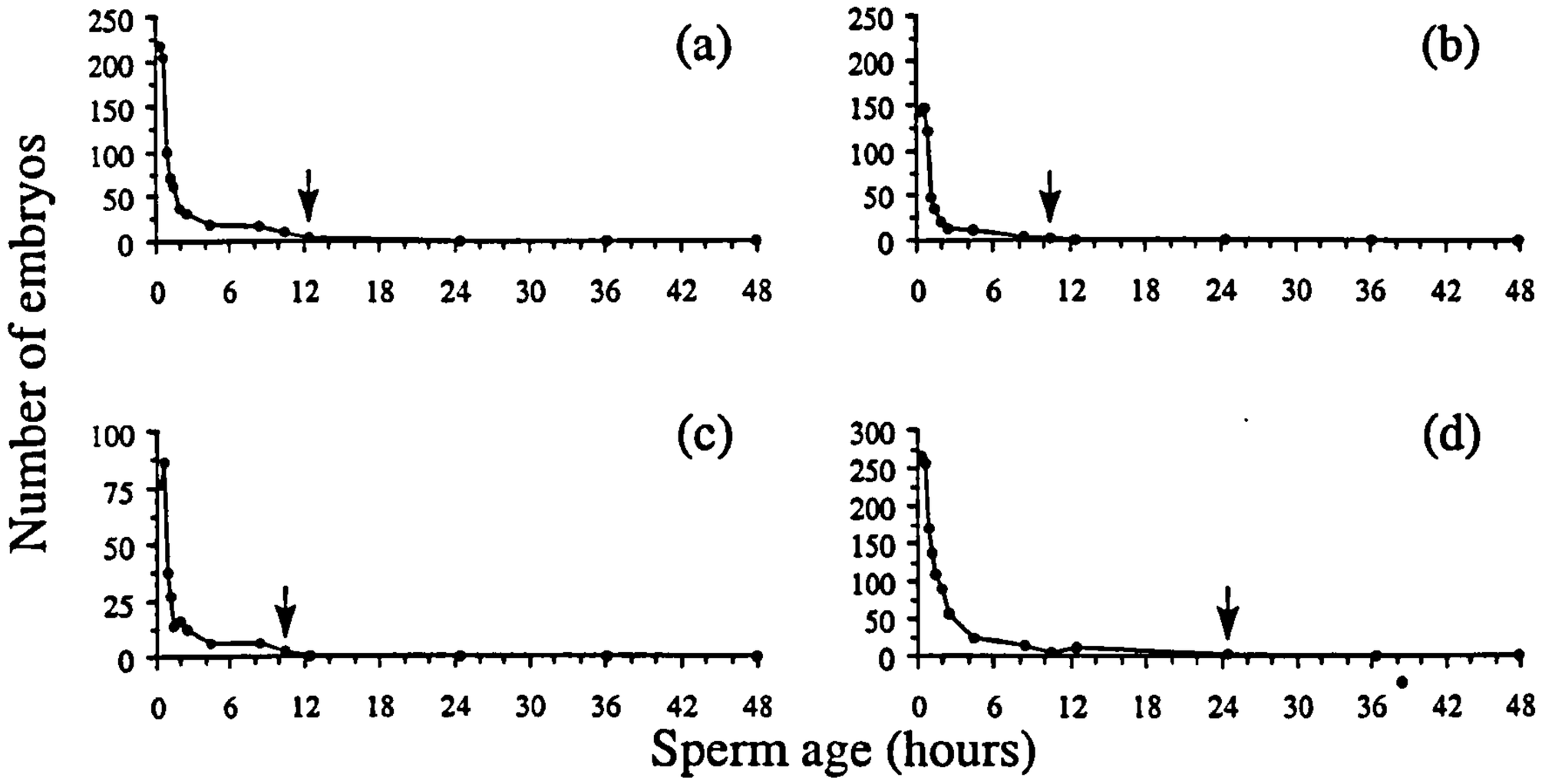

Figure 1: Experiment 1, diminution in number of embryos produced by colonies of C. hyalina as a function of increasing sperm age. Each graphic represent the results of individual experiments; (a) donor clone M_1, recipient clone A_1; (b) donor A_1, recipient E_1; (c) donor Q_1, recipient J_1; (d) donor Q_1, recipient D_1. The arrows indicate the oldest allosperm suspensions achieving fertilization.

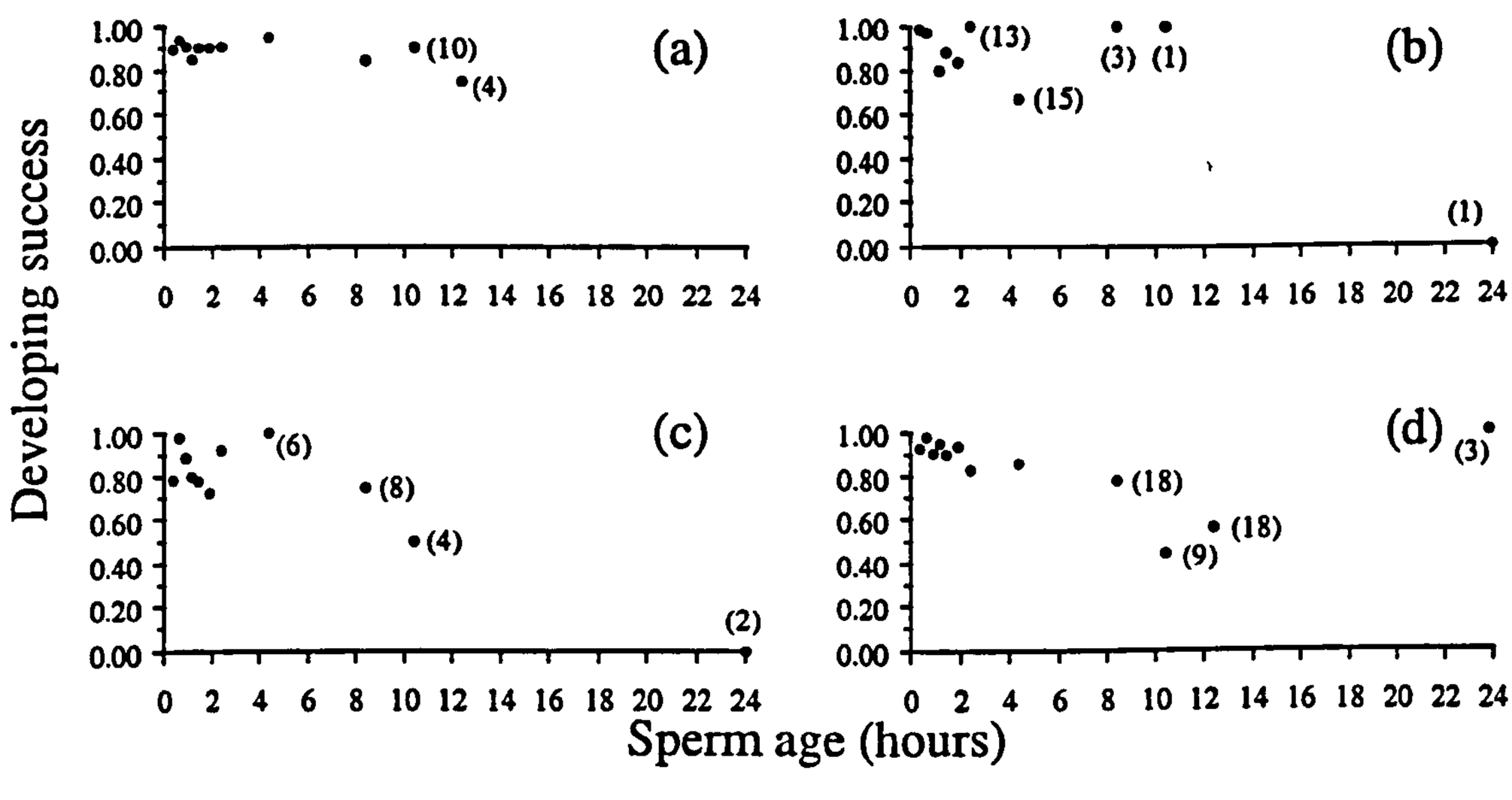

Figure 2: Experiment 1, relationship between proportion of oocytes becoming embryos by colonies of C. hyalina and sperm age. Each graph represents the results of individual experiments; (a) donor clone M_1, recipient clone A_1; (b) donor A_1, recipient E_1; (c) donor Q_1, recipient J_1; (d) donor Q_1, recipient D_1. Numbers in brackets represent number of oocytes followed and point without number correspond to number of oocytes larger than 10.

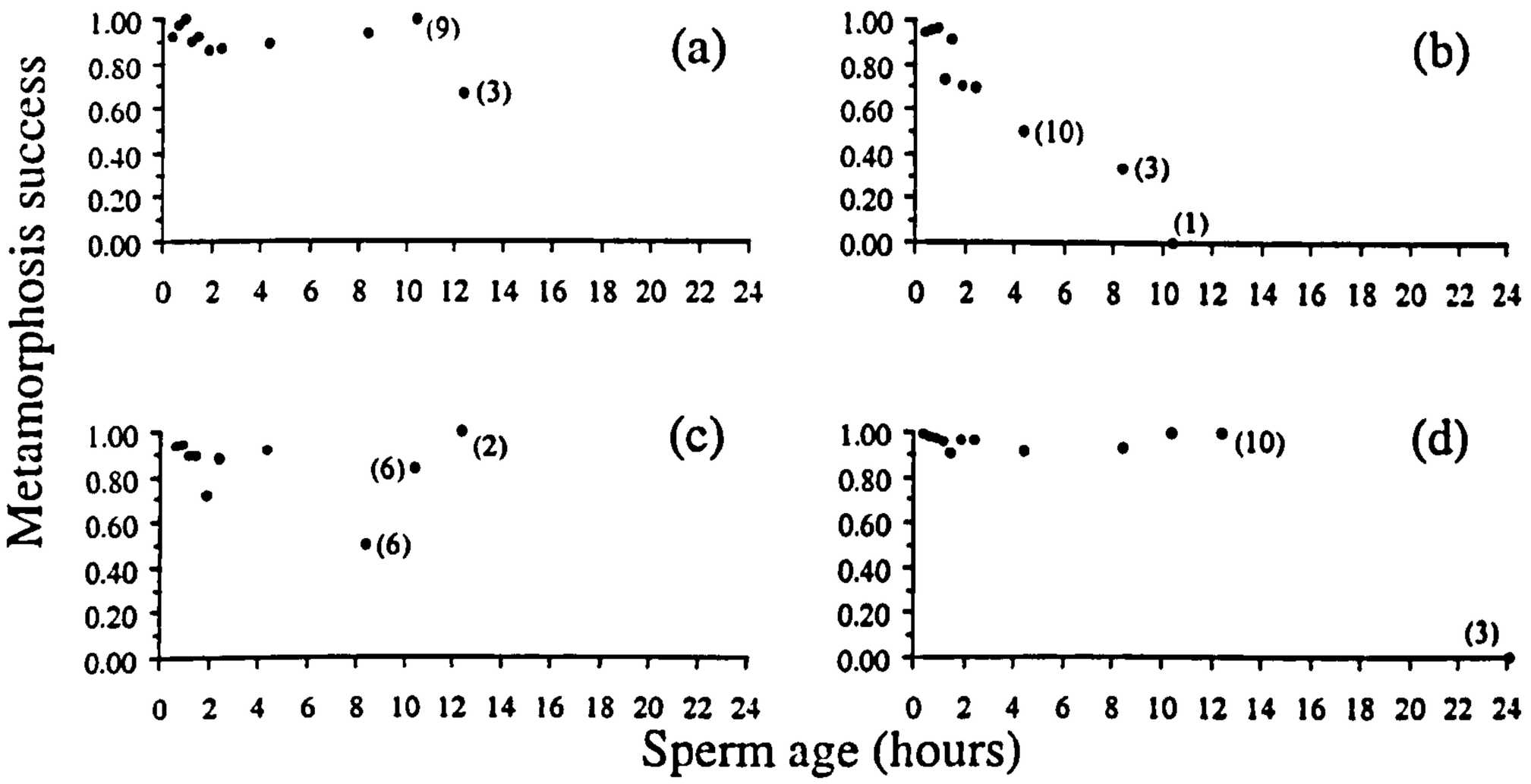

Figure 3: Experiment 1, relationship between proportion of embryos that metamorphosed into a new colony of C. hyalina and sperm age. Each graph represents the results of individual experiments; (a) donor clone M_1, recipient clone A_1; (b) donor A_1, recipient E_1; (c) donor Q_1, recipient J_1; (d) donor Q_1, recipient D_1. Numbers in brackets represent number of oocytes followed and point without number correspond to number of oocytes larger than 10.

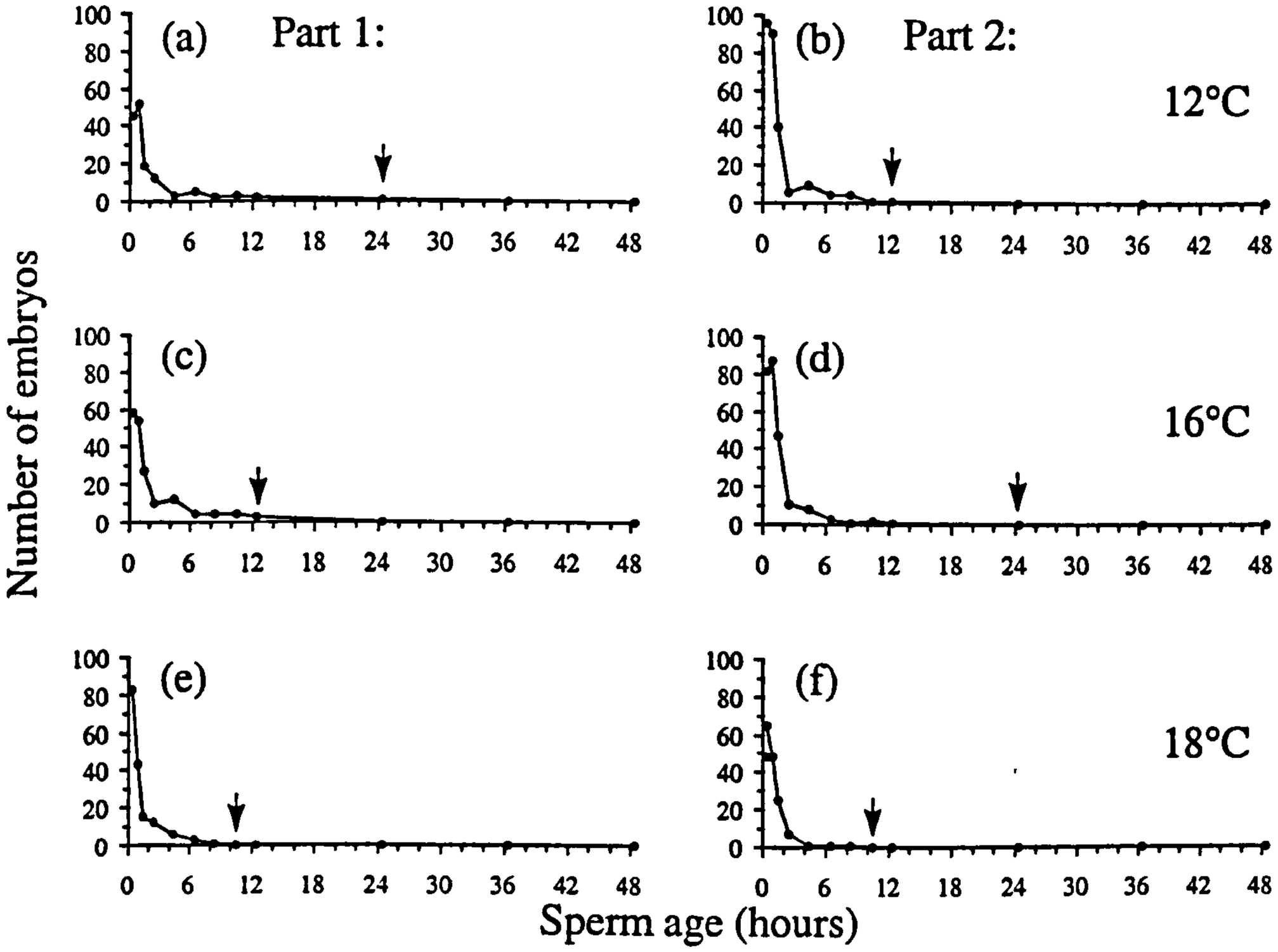

Figure 4: Experiment 2, diminution in number of embryos produced by colonies of C. hyalina as a function of increasing sperm age at three temperatures during the contact time between receptor colonies and the allosperm suspensions. Graphics on the left correspond to part 1, where receptor colonies from clone A_1 were exposed to allosperm from clone M_1. Graphics on the right correspond to part 2, were receptor colonies from the E_1 were exposed to allosperm from clone A_1. The arrows indicate the oldest allosperm suspensions achieving fertilization.

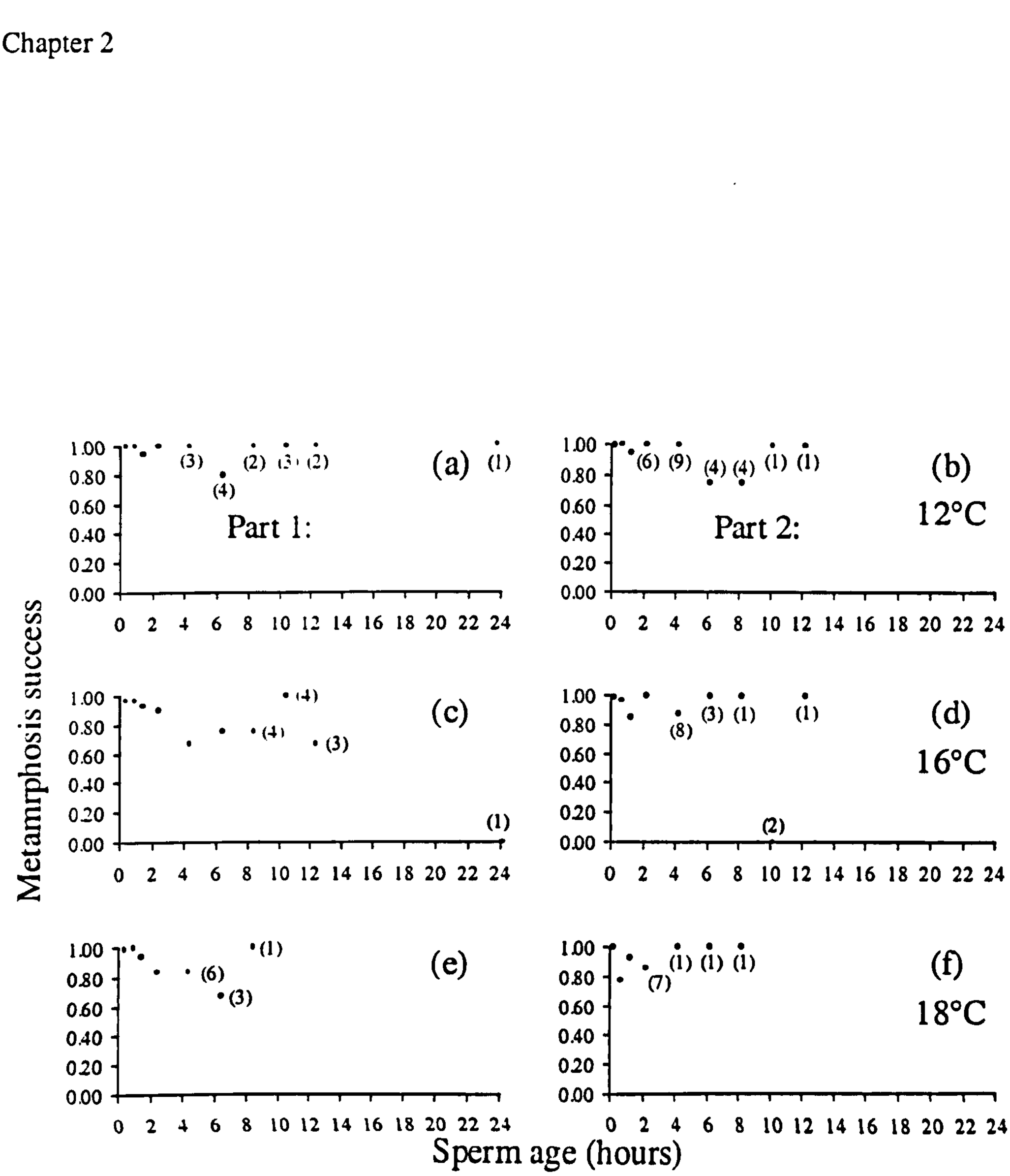

Figure 5: Experiment 2, relationship between proportion of embryos that settled and metamorphosed into a new colony of C. hyalina and sperm age at three temperatures during the contact between the receptor colonies and the allosperm suspensions. Graphs on the left correspond to part 1 (colonies from the A_1 were exposed to allosperm from clone M_1). Graphs on the right correspond to part 2 (colonies from the E_1 were exposed to allosperm from clone A_1). Numbers in brackets represent number of oocytes followed and point without number correspond to number of oocytes larger than 10.

Chapter 3
Brooding time, allosperm storage and intra-colonial sperm movement.

3.1 Introduction

Marine invertebrates whose sperm are released into the seawater but whose eggs are retained and fertilized internally are exposed to potential multiple mating. Captured sperm could reach and fertilize eggs or be moved to a storage site. Sperm storage occurs in many hermaphroditic invertebrates (Ghiselin, 1969), where it is usually associated with internal fertilization (Adiyodi and Adiyodi, 1988, 1990). Long term storage would assure continued fertility (Adiyodi and Adiyodi, 1988, 1990), and could be linked selection of sperm from a variety of sources allowing female choice. Sperm storage has been reported in most gastropods (Baur, 1988), in which it may be advantageous to mate with the first partner encountered, avoiding the high cost of locomotion associated with search for other mates (Baur, 1992).

To date, storage of exogenous sperm has been described in few marine invertebrates with transfer of aquatic sperm (Anderson, 1904; Holland, 1976; Miller, 1982; Bishop and Ryland, 1991; Todd et al., 1997). In gymnolaemate Bryozoa, aquasperm typically make their way through the coelomopore into the coelom of the maternal zooid and penetrate undischarged, partially-grown oocytes in the ovary (see Ryland and Bishop, 1993). Celleporella hyalina (L.) is a hermaphrodite gymnolaemate where fertilization takes place in retained ovulated eggs (i.e. initial sperm penetration before ovulation) which are then transferred to the corresponding brooding chambers, or ovicells, where the embryos obtain nutrition from the maternal zooid (Dyrynda and King, 1983). In Welsh C. hyalina embryos are observable within the coelom for 2-8 days (Cancino, 1983, 1988; D.J. Hughes, 1987) before being transferred to the ovicells. The duration of brooding time, from first appearance of the eggs in the coelom until larval release is about 3-4 wk (Cancino, 1983, 1988; D.J. Hughes, 1987). Similar brooding times of 13-19 days have been recorded for North American C. hyalina (McCartney, 1994), suggesting that brooding time is relatively constant in C. hyalina. Sperm are released through the male lophophore (see Chapter 4), and in recipient colonies uptake is via the protruded lophophores of autozooids (Silén, 1966; Temkin, 1994, 1996).

Presence of sperm inside male. female and feeding zooids has been interpreted as evidence of intra-colonial sperm migration (Marcus, 1938). Cancino (1983) mentioned the possibility of sperm storage in colonies of C. hyalina. He observed the presence of embryos and production of viable ancestrula in sexually mature colonies moved from the field to the laboratory and reared under reproductive isolation. To explain his findings, Cancino considered self-fertilization, parthenogenesis or sperm storage. As self-fertilization is rare in C. hyalina (see Chapter 7, Hoare et al., 1999) and the protocols of reproductive isolation used by Cancino were adequate, it seems that sperm storage was the most likely explanation for the appearance of embryos in the experimentally isolated colonies. Although parthenogenesis is a common phenomenon in invertebrate taxa (Hughes and Cancino, 1985, RN Hughes, 1989), no evidence of parthenogenesis has been published for C. hyalina.

The possibility of recording zygotes and brooded embryos in C. hyalina, together with the relatively constant duration of brooding provide a useful opportunity to investigate the possibility of storage of exogenous sperm (i.e. allosperm). Moreover, since self-fertilization is rare in the Welsh C. hyalina (see Chapter 7; Hoare et al., 1999) and sperm release can be induced by the dark-light treatment (see Appendix 2), allosperm dosage can be controlled experimentally. This offers the possibility of investigating in detail the brooding cycle in C. hyalina. The present study aims to use observations on cross-fertilized zygotes in C. hyalina to investigate the potential occurrence and duration of sperm storage. Moreover, within the same experiments this study aims to obtain information on the duration of the brooding cycle, fertilization success and sperm movement within the recipient colonies. Finally, observations were made on colonies collected in the field in order to estimate storage duration of sperm captured under natural conditions.

3.2 Materials and methods

3.2.1 Clones. sperm suspensions and rearing conditions

The experimental clones used in the present study (A_1, D_1, E_1, J_1, H_1, M_1, Q_1, A_2, H_2, T_2 and W_2) were established and maintained in reproductive isolation using the standard protocols (see Appendix 1). To assess sperm storage in wild material, colonies were collected during low tides in May and June of 1998 and May and June 1999 from the Menai Strait at Beaumaris (53°16'; 4°05'W) and Church Island (53°13'; 4°10'W).

Allosperm suspensions were obtained using a light/dark shift technique applied to colonies maintained in darkness overnight (see Appendix 2) and under standard culture conditions (see Chapter 2). Receptor colonies were exposed to 200 ml of selfsperm suspension or allosperm suspensions for 1-2 h depending on the experiment. Exposure to allosperm was conducted only when the sperm concentration was sufficient not to limit fertilization (see Appendix 2). The colonies were exposed to sperm suspensions in 300 ml glass vessels, and a few drops of R. reticulata were added to the vessel in order to enhance feeding activity. To avoid contamination by aerosol during the time the receptor colonies were in contact with the sperm suspensions, no aeration was used. The vessels bearing the receptor colonies with the sperm suspensions were placed in a water bath and the temperature maintained at 16°C. After exposure to allosperm suspensions, the colonies were cleaned with a 5 min rinse of FSW in order to remove any remaining allosperm. Colonies were then individually assigned to a 300 ml flat bottomed glass vessel closed with a perforated plastic cover (see Appendix 1). The colonies were fed every day using R. reticulata at concentrations of approximately 100 cells.μl^{-1} (see above). Water changes were carried out every second day and the colonies and the acetate substratum cleaned once a week (see Appendix 1). In preliminary trials, sexual compatibility among the experimental clones was investigated and only those proving to be compatible were used for the present experiments.

3.2.2 Experimental measurements

The observations of the colonies were conducted using a stereo microscope (Wild M4) and a cold light source. Drawings were used to record the information from the experimental colonies. The drawings were made using a camera lucida and a stereo

microscope and including the natural growing edge of each colony and the position of every visible female zooid. Stages of female ontogeny, from a clearly defined female bud to a female with a fully developed brood chamber or ovicell, were recorded. Since the colonies were continually growing, newly budded female zooids originating within and beyond the initial growing edge were added to the drawings every day. Once the last ovulated coelomic oocytes (hereafter oocytes) were recorded, the colonies were maintained under daily observation for two more weeks.

3.2.3 Sperm storage, brooding duration, brooding success and sperm movement

The appearance of the first and last oocytes in the coelom of the female zooids of each colony was recorded. The elapsed time between allosperm dosage and appearance of the first oocyte was considered as the minimum time required for pre-zygotic oocytes to become visible within the female coelom. The last oocytes to appear were considered to have been fertilized by stored sperm. In order to estimate duration of brooding period and brooding success in Experiment 1, 2, 3 and 5, 20 randomly chosen oocytes per colony were observed daily. The same measurements were made in 30 randomly chosen oocytes in Experiment 6. In Experiment 7, 20 random oocytes were followed in 6 different colonies (see Experiment 7 for details). Only oocytes completing the brooding cycle and leaving the colonies as larvae were included in the computation of brooding duration. The delay between cessation of exposure to allosperm and production of the last progeny, coupled with knowledge of the duration of the brooding cycle should indicate the effective duration of sperm storage. Abortion or fertilization failures were scored when developing oocytes disappeared without being transferred to the corresponding ovicells. Embryos disappearing from the ovicells without being released as larvae were considered to have been aborted. Thus, the percentage of oocytes successfully completing the brooding cycle was used as the basis for comparing treatments. To obtain larval settlement the standard settlement units were used (see Chapter 2). In all experiments, observations were conducted at daily intervals until the last ancestrula was noted in the settlement units. Information on the last settled larvae together with information on the brooding cycle was used as an alternative method for assessing sperm storage. Production of oocytes and brooding of embryos in female zooids situated beyond the original growing edge of the colony were ascribed to intra-colonial movement of allosperm. The position of such

embryos was recorded on the camera lucida drawings and the distance from the original natural growing edge calculated. In some cases, samples of DNA were extracted (see Chapter 5 for details) and used to assess paternity using published microsatellite markers (Hoare et al., 1998). Embryos sired by females budded beyond the original growing edge were expressed as a percentage of the total number of embryos sired by the entire colony. The last visible oocytes and the last settled larvae from the wild colonies were considered to have been fertilized by sperm that had penetrated the colonies before removal from the field. As in the laboratory-reared colonies, this information was then combined with brooding time and used to assess minimum duration of sperm storage.

In summary, the main questions of this study were: (1) is the duration of sperm storage affected by the experimental variables? (as described in each experiment below) (2) is the duration of brooding affected by the experimental variables? (3) is brooding success affected by the experimental variables? Statistical treatment included simple factorial analysis of variance (ANOVA) and paired-sample *t* test. Brooding success and sperm movement data were subjected to angular transformation (Sokal and Rolf, 1995). All statistical analyses were conducted using SPSS 8.0 data analysis program.

3.2.4 Experiment 1: maximum sperm storage, brooding time and sperm movement with one experimental allosperm dosage

Two virgin ramets of C. hyalina from each of 7 clones were exposed for 1 h to a sexually compatible allosperm suspension. In the first trial, ramets of clone A1 were used as the sperm donor and ramets of clones D_1, E_1, Q_1, H_1, J_1, M_1 and A_2 as sperm receptors. In the second trial ramets of clone Q1 were used as the sperm donor, and ramets of clones A_1, D_1, E_1, H_1, J_1, M_1, Q_1 and A_2 as allosperm receptors. After exposure to allosperm, the colonies were rinsed with FSW for 5 minutes. One of the two ramets was assigned to a 300 ml glass vessel filled with FSW (see Appendix 1) and maintained under reproductive isolation. The other ramet was assigned to a similar vessel together with a virgin clone-mate. This treatment was used to investigate if allosperm could be taken up by a colony, released again and then be captured by another colony. Two extra ramets from the same clones were used to control for self-fertilization. One colony was exposed to selfsperm suspension for 1 h, then rinsed with FSW for 5 min. The other colony was removed from

the stock culture and rinsed with FSW for 5 min. The control colonies were assigned to individual 300 ml glass vessels filled with FSW.

3.2.5 Experiment 2: maximum sperm storage, brooding time and sperm movement with two experimental allosperm dosages

Since exposure to allosperm induces production of sexual zooids (see Chapter 7), the present experiment aimed to investigate whether duration of both sperm storage and brooding time are different between virgin and non-virgin colonies. Since more female zooids will be present in colonies previously exposed to allosperm suspension than virgin colonies, a possible positive effect of female investment on sperm storage, brooding time and sperm movement are expected.

A virgin ramet from each of 5 different clones was used. Ramets of clone E1 were used as sperm donors and ramets of clones A_1, H_1, J_1, and Q_1 were used as sperm receptors. The receptor colonies were then exposed to a second allosperm suspension two weeks after the last oocyte resulting from the first allosperm dosage had been observed. Receptor colonies were exposed to the first and second sperm suspensions for 1 h and the colonies maintained in 300 ml glass vessels (see Appendix 1). As a control for self-fertilization, one ramet of each clone was maintained in reproductive isolation until the end of the experimental period.

3.2.6 Experiment 3: the effect of colony size on maximum sperm storage, brooding time and sperm movement

Normally in C. hyalina the number of reproductive zooids is positively correlated with the number of autozooids in the colony (Cancino, 1983). However, in reproductive isolation production of female zooids is largely depressed in comparison with colonies similar in size exposed to allosperm (see Chapter 7) growing on the field (personal observation). Since large virgin colonies exposed to allosperm should be potentially capable of allocating more energy to progeny than smaller ones, it is expected that colony size could affect sperm storage, brooding time and sperm movement.

Three ramets of C. hyalina from each of 3 different clones were used (M_1, E_1 and Q_1). From each clone, small (ca. 600 basal zooids), medium (ca. 1200 basal zooids) and large

(ca. 2500 basal zooids) ramets were used. In order to avoid differential allosperm uptake related to colony size, the ramets were exposed individually to a standard concentration of allosperm suspension in different 300 ml vessels. The ramets were exposed to the allosperm suspension for 2 h, rinsed with FSW for 5 minutes and then assigned to separate 250 ml cultured vessels (see Appendix 1). One ramet from each clone was maintained in reproductive isolation as a control for self-fertilization.

3.2.7 **Experiment 4:** maximum sperm storage and direction of sperm movement

In the present experiment sperm storage and the direction of sperm movement were studied using a modified receptor colony, from which most of the central zooids had been removed by scraping from the experimental substratum, leaving a ring of 20-25 rows of basal zooids per colony (Plate 1). The annular colonies were maintained in reproductive isolation for one month, by which time they had developed two meristems, the inner one growing centripetally and the outer one centrifugally. The experiment was run twice first using 3 annular colonies of clone F_1 as receptors. One annular colony was exposed to allosperm from clone A_1, the second to allosperm from clone E_1, and the last ring maintained under reproductive isolation as control for self-fertilization. In the second run, annular colonies of clone J_2 were used as receptors and clones A_1 and E_1 were used as sexually compatible allosperm donors. The same observations as in the previous experiment were made. but special care was taken to differentiate progeny produced in different areas in the receptor colonies: beyond the external growing edge; beyond the internal growing edge and within the two growing edges. In order to obtain DNA for parental analysis late embryos were removed from the ovicells in each area of the receptor colonies. Twenty embryos were removed from each colony, 5 being removed from ovicells budded beyond the outer growing edge, 5 from ovicells budded beyond the inner growing edge and 10 from ovicells budded inside the initial growing edges. In the last group, 5 had been brooded in females already present at the moment that allosperm dosage took place and 5 embryos brooded in ovicells not visible at that time. A clean, sterilized needle was used to carefully crack the ovicells and then late embryos were removed by suction using a Gilson® pipette. To avoid trapping embryos on the walls of the pipette tip, large diameter tips were used. Collected embryos were placed into wells of an HLA plate

and then placed in Eppendorf micro tubes with 10 µl of TE. For long term storage, the micro tubes bearing the samples were maintained at -80°C.

3.2.8 Experiment 5: influence of female investment on maximum sperm storage and brooding time

The present experiment was designed to investigate whether gender allocation influences the duration of sperm storage and brooding time. From cultures kept in reproductive isolation, three female biased clones were selected (T_2, H_2 and W_2) each having about 20 times more female zooids per unit area than normal clones (personal observations). Six ramets of each clone were used. In addition, six ramets of C. hyalina from of each three normal clones, D_1, E_1 and M_1 were used. Three ramets of each clone were exposed for 2 h to similar concentrations of compatible allosperm suspension (A_1). The remaining 3 ramets were maintained in reproductive isolation as control colonies. Observations similar to those described in Experiments 3 and 4 were taken here until no more progeny were detected in the colonies. Observations of progeny brooded beyond and within the original colonial edges were assessed in one ramet of each clone

3.2.9 Experiment 6: influence of reproductive history on maximum sperm storage and brooding time

In the present experiment, the possible effect of cumulative reproductive activity on maximum duration of sperm storage, brooding time and fertilization success was investigated. Colonies exposed for longer periods to allosperm suspensions had experienced more brooding cycles than colonies exposed for less time. To obtain females with different cumulative reproductive activity, ramets from 3 clones were exposed continuously for 1, 2 and 4 months to allosperm suspension in PET bottles (see Appendix 1). Thus, most of the female zooids present in the ramets exposed for 4 months to allosperm had experienced more brooding cycles than those exposed for 1 month. At the same time. control ramets of the same clones were maintained in reproductive isolation in separate PET bottles. After exposure to allosperm, colonies were maintained in reproductive isolation until the last oocytes were detected. These colonies, together with control ramets, were exposed simultaneously to compatible allosperm suspensions. Observations were made as in Experiments 3-5, but special care was taken in order to

focus on equivalent female zooids present when the first phase of exposure to allosperm was terminated. Observations were focused on equivalent female zooids in all the colonies i.e. those not budded beyond the colonial growing edge at the time when colonies were exposed to allosperm. Camera lucida drawings were used to identify the original growing edge and the female zooids. A trace made with a waterproof pen onto the surface of the acetate sheet was used to indicate the original growing edge. No evidence of deleterious effects of the waterproof pen on the colonies was detected in previous trials.

3.2.10 Experiment 7: maximum sperm storage in wild colonies

In the present experiment, sperm storage was studied in wild colonies of C. hyalina growing on Fucus serratus, one of their natural substrata. Pieces of algal tissue each bearing a reproductive colony (ca. 1000-1500 basal zooids) were cut from the frond and placed separately in small 10 ml plastic Petri dished filled with FSW. They were maintained in total darkness in order to enhance degeneration of the algal tissue. Each day the water in the Petri dishes was carefully removed with a plastic pipette and replaced with clean FSW. During this process, rotten pieces of algal tissue were removed from the Petri dishes using a pipette, a soft artist's brush and a fine forceps. Special care was taken to avoid touching the colonies. Thus, by the end of the second week most of the algal tissue was removed from the base of the colonies. Once the colonies were no longer attached to their substrata, observations were made on the living colonies. When the colonies were still attached to the algal tissue, however observations of oocytes were carried out using a cold light source placed directly beneath the colonies. All observations were made in the Petri dishes and the colonies carefully manipulated with a soft artist's brush. These observations were made in 12 colonies for each month (May and June) and locality (Beaumaris (53°16' N; 4°05' W) and Church Island (53°13'N; 4°10'W)). Length of the complete brooding cycle and brooding success were assessed by following 20 oocytes in 6 colonies. These colonies were collected during the lowest tides in May of 1999; 3 colonies were from Beaumaris and 3 from Church Island.

3.2.11 Experiment 8: sperm storage in non-reproductive colonies

Experiments 1-5 used sexually mature colonies. In the present experiment, the possibility of sperm storage through precocious mating was investigated using two approaches. In the

first, two small sections of acetate sheet bearing between 30-40 basal zooids were removed from the natural growing edge from ramets of each of 6 different clones (A_1, M_1, E_1, Q_1, J_1 and D_1). The sections were attached to larger 4.5 x 7.5 cm pieces of acetate sheet using a small amount of cyanoacrylate adhesive (see Appendix 1). Special care was taken to choose sections only bearing basal autozooids without any fully developed reproductive zooids or early reproductive buds. Once the new ramets had grown onto the experimental substratum, the initial small piece of plastic was removed and the remaining basal zooids reduced to 10 feeding autozooids (i.e. autozooids with a fully developed feeding apparatus) in number (Plate 2a). After two days, 2 ramets from the 6 different clones were exposed simultaneously to compatible allosperm suspension for 2 h. In this experiment a mixture of allosperm from 3 clones was administrated to each receptor clone (A_1, E_1, and M_1). One ramet from each receptor clone was then assigned to reproductive isolation (Treatment 1) and the remaining ones exposed every day to compatible allosperm suspensions from clone N_2 (Treatment 2). This second treatment was included in order to compare sexual allocation between colonies exposed once and those exposed daily to allosperm suspension (see Chapter 7 for results on that topic). Data also were used to estimate sperm storage duration. All the experimental colonies were maintained under standard culture conditions (above) for 10 weeks. Colonies were observed at daily intervals and presence of oocytes and their position in the receptor colonies noted in camera lucida drawings. All the progeny from treatment 1 were collected for fingerprinting, however from Treatment 2 only the first 15 produced embryos were collected.

In the second approach, a similar experimental protocol was followed, but the small receptor colonies were replaced by newly settled colonies. These colonies were obtained from 4 controlled matings. Individual larvae were induced to settled on conditioned acetate sheet and then a small section bearing a newly settled colony was trimmed and attached to a second acetate sheet as described above. Once the newly settled colonies were between 3-4 basal feeding autozooids in size (Plate 2a), they were each exposed to an allosperm suspension. From each mating, two of those small colonies were exposed for 2 h to a mixed allosperm suspension (A_1, M_1, E_1). Then, one colony from each pair was assigned to reproductive isolation (Treatment 1) and the other one exposed every day to compatible allosperm suspensions from clone N_2 (Treatment 2). An extra small colony

from each mating was used as a control for self-fertilization and maintained under reproductive isolation. Two experimental runs were made using the same paternal clones and the same protocols. In the first run no progeny were collected for fingerprinting from the Treatment 2. however all the progeny from Treatment 1 were collected. In the second run, samples were taken from both treatments. The first visible embryos close to the original zooids were collected from Treatment 2 and all the embryos produced in Treatment 1 were collected. For fingerprinting samples were stored as described in Experiment 4.

3.3 Results

3.3.1 Experiment 1: maximum sperm storage, brooding time and sperm movement with one experimental allosperm dosage

Oocytes. embryos and larvae were not produced by receptor colonies exposed to autosperm suspensions or by those maintained in reproductive isolation. The same results were found in receptor colonies placed in the same experimental vessel with a clone-mate previously exposed to sexually compatible allosperm suspension. Different stages of embryogenesis from ovulated coelomic oocyte to late embryo were found in all the receptor colonies exposed to sexually compatible allosperm suspensions. Timing of the appearance of the first and last ovulated coelomic oocytes and settled larvae in trials 1 and 2 are summarized in Table 1. In general, the first oocytes were visible inside the females' bodies during the first week after exposure to allosperm suspension. However, the last oocytes were visible from the third to fifth week after allosperm dosage. Settlement occurred from the third to ninth week after the allosperm dosage.

The duration of ovulated coelomic oocytes and embryos inside the females' body and brood chamber respectively are summarized in Table 2. In general, oocytes were visible in the female bodies from 1 to 8 d, and embryos inside the ovicells from 12 to 23 d. High survivorship of embryos (ca. > 98%) confirmed by permanence in the females' body and in the ovicells was found (Table 2). Thus, duration of the total brooding cycle ranged from about 2 to 4 weeks.

Proportion of progeny inside the receptor colonies is shown in figure 1. Most of the progeny (ca. >90%) were produced by female zooids situated within the initial colonial growing edge at the moment that exposure to allosperm took place. However, progeny were also produced by female zooids budded beyond the initial growing edge (Fig. 1). The proportion of progeny produced within the original growing edge was similar between females present originally and budded later.

3.3.2 Experiment 2: maximum sperm storage, brooding time and sperm movement with two experimental allosperm dosages

Oocytes, embryos and larvae were not produced in the control colonies, confirming the absence of self-fertilization (see Chapter 4). Data on appearance of the first and last oocytes and settled larvae are summarized in Table 3 and on duration of the brooding cycle in Table 4. ANOVA showed no significant differences in timing of appearance of the last oocyte attributed to dosage, clone, or their combined effect (F=1.13; DF=1; SS=4.90; MS=4.90; P>0.05 for allosperm dosage; F=0.51; df=4; SS=75.40; MS= 18.85; P>0.05 for clone and F=0.44; DF=5; SS=80.30; MS= 16.06; P>0.05 for the combined effect). There were significant differences in duration of the complete brooding cycle attributed to the allosperm dosage (F=4.22; DF=1; SS=363.01; MS=363.01; P<0.05). On average, the duration of the brooding cycle was longer for progeny from the second allosperm dosage than for progeny from the first one (Table 4). However, there were no significant differences attributed to clone and the combined effect of clone and dosage (F=0.45; DF=3; SS=115.27; MS= 38.42; P>0.05 for clone, and F=1.39; DF=4; SS=478.28; MS= 119.57; P>0.05 for the combined effect). Survivorship of embryos was high (ca. > 99%) after allosperm dosage, and no failure occurred regarding permanence in the females' body and in the ovicells (Table 4). After exposure to the first and second allosperm dosage, most of the progeny (ca. >99%) were produced by female zooids within the colonial growing edge at the moment that exposure to allosperm took place. However, a small percentage of the progeny was produced by female zooids budded beyond this edge (Fig. 2). After the first allosperm dosage, similar percentages of progeny were produced by females present within the original colonial edges and by females budded later in the same area (Fig. 2a). However, after the second allosperm dosage, on average more embryos were produced by females already present in the colonies than in newly budded female zooids (Fig. 2b). On average a higher proportion embryos were produced by females present already in the colonies after the second allosperm dosage than after the first dosage, although the difference was not statistically significant (t=2.19; DF=4; P>0.05).

3.3.3 Experiment 3: the effect of colony size on maximum sperm storage, brooding time and sperm movement

As in the previous experiments. absence of progeny in the control colonies confirmed the absence of self-fertilization. Data on the appearance of the first and last oocytes and settled larvae for different colonial sizes are summarized in Table 5. The duration of brooding is summarized in Table 6. ANOVA showed no significant differences in time of appearance of the last oocyte attributed to colony size (F=1.46; DF=2; SS=19.11; MS=9.56; P>0.05) However, appearance of the last oocyte was significantly affected by clone (F=21.45; DF=2; SS=289.44; MS=140.22; P<0.001) and colony size combined with clone (F=11.47; DF=4; SS=299.56; MS=74.89; P<0.001). ANOVA showed significant differences in duration of the complete brooding cycle (F=4.73; DF=2; SS=40.82; MS=20.41; P<0.05, for colony size: F=5.34: DF=2: SS=49.92; MS=24.96; P<0.05, for clone, F=4.86; DF=4; SS=90.76; MS=22.69; P<0.05, for the colony size combined with clone). On average, the brooding time was longer in larger than in medium and smaller colonies (Table 6). Brooding success in the females' body and over the complete cycle always exceeded 91%. The proportions of progeny within different areas of the receptor colonies are shown in Figure 3. As in the previous experiments, most of the progeny (ca. >90%) were produced by females within the initial growing edge. In small colonies, only a small proportion of the progeny (ca. <35%) were produced in these zooids. However, in medium and large colonies most of the progeny (ca.>65%) was produced in female zooids present in the colonies at the time of sperm dosage. ANOVA showed significant differences in the proportion of progeny found in ovicells present within the initial growing edges associated to colony size and a significant size by clone interaction (F=13.19; DF=2; SS= 0.41; MS= 0.21; P<0.05, for colony size; F=6.77; DF= 4; SS= 0.42; MS= 0.11; P<0.05, for colony size combined with clone). However, no significant differences were found associated with clone (F=0.35; DF=2; SS= 0.01; MS= 0.001; P>0.05). On average, more of the progeny was found in females present within the original growing edges in medium and larger colonies than in the smaller ones (Fig. 3).

3.3.4 Experiment 4: maximum sperm storage and direction of sperm movement

Since progeny were not found in the control colonies, this experiment confirms as did the previous experiments, the absence of self-fertilization in Welsh C. hyalina. Results for the

appearance of the first and last ovulated coelomic oocytes and settled larvae are summarized in the Table 7. The temporal duration of the brooding and brooding cycle is given in Table 8. The spatial distribution of the progeny in the receptor colonies is shown in Figure 4. Most of the progeny (ca. >90%) was produced by female zooids present within the colonial growing edges at the moment that allosperm dosage took place. However, a small percentage of the progeny was produced by female zooids budded centripetally and centrifugally beyond the original growing edges. On average the distance of these progeny from the original growing edge was 300 μm (SD= 45; N= 30 for progeny budded cetripetally) and 400 μm (SD= 45; N= 30 for progeny budded centrifugally). Fingerprint analyses showed that all the progeny produced by the receptor colonies, brooded within the original growing edges or beyond them, were sired by the corresponding allosperm donors.

3.3.5 Experiment 5: influence of female investment on maximum sperm storage and brooding time

No progeny were found in the control colonies, confirming as in the previous experiments, the absence of self-fertilization. Results for the appearance of the first and last ovulated coelomic oocytes and settled larvae are summarized in Table 9. Temporal duration of the brooding cycle and brooding success are given in Table 10. ANOVA showed no significant differences in time of appearance of the last coelomic oocyte between female biased and normal clones ($F=0.17$; $DF=1$; $P>0.05$). On average, the length of brooding was similar between female biased and normal clones ($F=0.33$ $DF=1$; $P>0.05$). Values of brooding success were maximum, with no apparent failure to develop in the female's body or in the ovicells. The proportions of progeny within the receptor colonies are shown in Figure 5. On average, a significantly higher proportion of the progeny were produced by female zooids present within the colonial growing edge when allosperm dosage occurred in the female biased clones than in the normal ones ($F=19.13$; $DF=1$; $P<0.05$). However, on average a significantly higher proportion of the progeny were produced in newly budded female zooids in the normal clones than in the feminized ones ($F=11.84$; $DF= 1$; $P<0.05$). Similarly, on average a significantly higher proportion of the progeny was produced by female zooids budded beyond the initial colonial edge in normal clones than in female biased clones ($F=8.86$; $DF=1$; $P<0.05$)

3.3.6 Experiment 6: influence of reproductive history on maximum sperm storage and brooding time

No progeny were found in the control colonies, so this experiment confirmed, as did the previous experiments, absence of self-fertilization. Results for the appearance of the first and last oocytes and settled larvae are summarized in Table 11. Duration of the brooding cycle and brooding success are given in Table 12. ANOVA showed no significant differences in time of appearance of the last coelomic oocyte associated with cumulative reproductive activity ($F=0.23$; $DF=2$; $SS=2.89$; $MS=1.44$; $P>0.05$) and reproductive activity combined with clone (and $F=4.80$; $DF=4$; $SS=120.44$; $MS=30.11$; $P>0.05$). However. a significant effect of clone was found ($F=9.36$; $DF=2$; $SS=117.56$; $MS=58.78$; $P<0.05$). ANOVA showed significant differences in duration of the complete brooding cycle attributed to cumulative reproductive activity ($F=12.01$; $DF=2$; $SS=135.63$; $MS=67.81$; $P<0.0001$) and the combined effect with clone ($F=6.45$; $DF=4$; $SS=145.64$; $MS=36.41$; $P<0.001$). On average, a longer brooding cycle was found in colonies having had greater activity. There was no significant effect of clone on the duration of the complete brooding cycle ($F=0.95$; $DF=2$; $SS=10.77$; $MS=5.39$; $P>0.05$). Despite brooding success always being over 70%, ANOVA showed significant differences associated with cumulative reproductive activity ($F=35.50$; $DF=2$; $SS=1.46$; $MS=0.73$; $P<0.001$) and reproductive activity combined with clone ($F=17.90$; $DF=4$; $SS=1.51$; $MS=0.38$; $P<0.001$). On average, brooding success was lower in colonies that had experienced greater reproductive activity than in colonies experiencing less reproductive activity. A no significant effect of clone on brooding success was found ($F=1.31$; $DF=2$; $SS=0.006$; $MS=0.03$; $P>0.05$). The proportions of progeny within the receptor colonies are shown in Figure 6. On average the proportion of progeny produced by female zooids present in the colonies at the time of sperm dosage increased with the age of the female zooids. The results indicated that colonies experiencing 4 months of reproductive activity had a significantly higher proportion of progeny produced by female zooids present in the colonies than colonies with 2 and 1 months of reproductive activity. ($F=83.61$; $DF=2$; $SS=0.34$; $MS=0.17$; $P<0.005$, for reproductive activity; and $F=52.26$; $DF=2$; $SS=0.34$; $MS=0.08$; $P<0.0005$, for reproductive activity combined with clone). No significant effect of clone on the proportion of progeny produced by female zooids present in the colonies at

the time of allosperm dosage took place was found (F=0.90; DF=2; SS= 0.004; MS= 0.002: P>0.05). The proportion of progeny produced by females budded later inside the original growing edges was significantly higher in colonies with 1 month of reproductive history than colonies with 2 and 4 months of reproductive activity (F=45.85; DF= 2; SS= 0.34: MS= 0.17; P<0.005) with a significant effect of reproductive activity combined with clone (F=23.59; DF= 4; SS= 0.35; MS= 0.09; P<0.05). There was no significant effect of clone in the proportion of progeny sired by female zooids budded later inside the original growing edge (F=0.376; DF= 2; SS= 0.01; MS=0.005; P>0.05). Progeny were produced beyond the initial growing edge in all the treatments, and no significant differences were found between them (F=2.02; DF=2; SS=0.03; MS=0.02; P>0.05, for reproductive activity; F=1.12; DF=2; SS=0.02; MS= 0.01; P>0.05, for clone; F=0.05; DF= 4; SS= 0.05; MS= 0.01; P>0.05 for the combined effect).

3.3.7 Experiment 7: maximum sperm storage in wild colonies

Oocytes were visible inside the female zooids in all the wild colonies from the day that collection took place. Appearance of the last ovulated coelomic oocytes visible in the body of the female zooids and information about the last newly settled larvae is given in Table 13. In general the last ovulated coelomic oocytes were visible inside the females' body from 3 to 17 d (1998), and from 2 to 23 d (1999) after colonies had been placed in reproductive isolation. On the other hand, the latency to settlement ranged from 16 to 36 d in 1998 and from 16 to 37 d in 1999. Information on brooding cycle length and brooding success are given in Table 14. Oocytes were visible in the female's body from 1 to 5 d and from 12 to 23 d in the brooding chamber. There was no significant difference in total length of the brooding cycle between localities (F=0.024; DF=1; P>0.05). Total brooding success was high (ca. >75%) and no significant difference between localities was detected (F=2.33; DF=1; P>0.05).

3.3.8 Experiment 8: sperm storage in non-reproductive colonies

3.3.8.1 From ancestrula

In the first trial, the first visible oocyte was found during the second week. By this time the colonies were about 41.75 basal zooids in size (SD=4.44; N=4) and with 90% of zooids

active (SD=3.9; N=4). Progeny were found in 3 of the 4 colonies in Treatment 1 (Table 15).

In the second trial, the first visible oocyte inside the female zooids was found in the second week when the colonies were about 38.50 basal zooid in size (SD=4.50; N=4) with 88% of zooids active (SD=2.95: N=4). As in the first run, progeny was found in 3 of the 4 colonies exposed to allosperm suspension. As in the previous trial, no progeny was found in the control colonies. Fingerprints showed that the complete pool of progeny was sired by allosperm present in the cocktail suspension. In Treatment 2, embryos were produced in the 4 colonies (Table 16), being in average the 30 % (SD=0.08; N=4) of the first 10 embryos fathered by sperm presents in the cocktail. The rest of the progeny were fathered by allosperm supplied after the second day. In control colonies, embryos and larval settlement were not found. Progeny produced by colonies exposed to allosperm were located in ovicells placed on top, or close to the initial zooids.

3.3.8.2 From 10 autozooids

In the first trial, the first oocytes were visible by the middle of the second week in 5 out of 6 of the colonies exposed to allosperm suspensions. By this time the colonies were about 64 basal zooids in size (SD= 5.74; N=6) and with 87% of their zooids active (SD=3.80; N=6). By the third week oocytes were visible in all the colonies when the colonies, which were about 127 zooids in size (SD=10.66; N=6) and with 89%.of their zooids active. Oocytes, embryos and larval settlement were present only in the colonies exposed to allosperm suspensions. In control colonies maintained under reproductive isolation during the same period of time oocytes, embryos and settlement were not found. The number of progeny produced per colony in Treatment 1 ranged from 0 to 5, all being fathered by allosperm present in the cocktail (Table 17). In Treatment 2, the 28 % of the first progeny budded close to the initial zooids were fathered by allosperm offered on the first day. The rest of the progeny were fathered by allosperm offered from the second day onwards (Table 18).

In the second trial, similar results were found. The first ovulated coelomic oocytes were found one week after exposure to allosperm when the colonies were about 32 basal zooids

in size (SD=4.43; N=6) and in which 83% of zooids were active (SD=9.49; N=6). By the end of the third week oocytes were present in the 6 colonies exposed to allosperm suspension, when the colonies were about 166 basal zooids in size (SD=18.20; N=6) and with 76% of zooids active (SD=7.25%; N=6). However, by the same time in the control colonies, oocytes were not visible. In the first treatment colonies produced from 0 to 3 progeny. Fingerprinting showed that all the progeny were fathered by sperm present in the cocktail offered during the first day. In Treatment 2, the 18 % of the first progeny budded close to the initial zooids were fathered by allosperm offered on the first day, the rest were fathered by allosperm offered from the second day onwards (Table 16).

3.4 Discussion

3.4.1 Self-fertilization

The results of the present study show prevalence of out-crossing in laboratory reared colonies of C. hyalina. This is supported by absence of visible oocytes, embryos and production of larvae in all the control colonies maintained in reproductive isolation (Experiments1-6 and 8). Moreover, absence of new visible oocytes within the female coelom 6 weeks after exposure to allosperm, supports the idea that once the maximum duration of sperm storage is reached, autosperm are not subsequently used to achieve more fertilizations. This is also supported by observation on wild colonies. Although colonies were still alive and had a high percentage of active zooids (> 60%, personal observation), production of new oocytes and embryos ceased about 2 weeks after being placed in reproductive isolation. Fingerprinting of colonies mated in the laboratory showed that only one of the 67 offspring was attributable to self-fertilization (ca. 1.5 % of the scored progeny). This corresponded to the offspring of a small 10-zooid colony exposed to a mixture of 2 different allosperm suspensions and autosperm suspensions (Experiment 8). Progeny were not produced by the corresponding control ramet maintained under reproductive isolation and then exposed to autosperm suspension throughout the experimental period. This suggests that the putative mechanism by which females control paternity and avoid self-fertilization may be overcome in the presence of high concentrations of allosperm. as those used in the present study. Female control of paternity through selection of sperm has been reported in another colonial marine organism with internal fertilization (Bishop, 1996; Bishop et al., 1996). It seems possible that a similar mechanism has evolved in C. hyalina to block fertilization by autosperm. Self-fertilization has been reported previously in C. hyalina (Hunter and Hughes, 1993c). However, the colonies were cultured in isolation and produced embryos and larvae with low viability. In the present study the only larva assigned to self-fertilization (Experiment 8) was removed from the corresponding ovicell before larval release took place and so its viability could not be assessed. The observed prevalence of out-crossing corroborates information generated throughout this dissertation and information generated in different studies with Welsh C. hyalina (Hoare et al., 1999). An important advantage of this finding in the context of the present Chapter is that it facilitates interpretation of the experimental results. In the absence of

self-fertilization and without contamination by unwanted allosperm, the complete pool of progeny should be fathered by the allosperm donor. Thus duration of sperm storage could reliably be assessed from the time of the last oocytes or settled larvae.

3.4.2 First ovulated coelomic oocytes

Appearance of the first ovulated coelomic oocytes (ca. 40 µm) from 2 to 6 days after allosperm dosage (Experiments 1-7) suggests that once their development is triggered by allosperm. pre-zygotic egg cells take this period to attain visible size. If allosperm are indeed responsible for triggering oocyte growth, appearance of the first oocytes in similar times after the first and second allosperm dosage (Experiment 2) suggests that activation of pre-zygotic egg cells was independent of previous exposure to allosperm. Time required to produce first visible oocytes after allosperm dosage, seems to be independent of colony size (Experiment 3), female investment (Experiment 5) and colonial reproductive history (Experiment 6). Presence of visible oocytes in wild colonies from the day of collection (personal observation), suggests that these colonies had already been exposed to allosperm suspensions when collected.

3.4.3 Last ovulated coelomic oocytes

Appearance of the last oocytes inside the female body 3 to 5 wk (Experiments 1-6) after the allosperm dosage strongly suggests that C. hyalina is capable of long term sperm storage. Lack of any difference in timing of the last oocytes produced by colonies exposed to one and two allosperm dosages (Experiment 2) suggests duration of sperm storage was independent of previous exposure to allosperm. Similarly, results of the present study suggest that maximum duration of sperm storage is independent of colony size (Experiment 3), female investment (Experiment 5) and past reproductive activity (Experiment 6). These results suggest that viability of stored sperm is a simple function of time and dependent on receptor attributes as in terrestrial gastropods, where the viability of stored sperm is influenced by the length of the aestivation period (Raut and Ghose, 1982).

Results from the wild colonies (Experiment 7), showed that the appearance of the last oocytes was about one week earlier than in colonies exposed to controlled allosperm

suspensions. This difference could be explained if allosperm were not available to the colonies during the day of the collection or during previous days. However, as the colonies were transported in plastic bags from the field to the laboratory it is highly probable that they were exposed to high allosperm suspensions during transit. Possibly, although allosperm were available for fertilization, stress incurred during transit had deleterious effects on newly captured sperm. Thus, progeny produced by wild colonies brought into the laboratory may have been sired by previously undisturbed stored sperm. As the laboratory propagated colonies were exposed to a non-limiting concentration (see Appendix 2) of fresh sperm, it seems reasonable to suppose that sperm ageing and sperm dilution could also explain differences in appearance of the last oocytes. In C. hyalina fertile half life is about 1 hour (see Chapter 2), sperm concentration has a significant effect on fertilization success (see Appendix 2), while sperm capture is affected by colonial physiology and ambient hydrodynamic regime. Therefore, the chance of wild colonies obtaining the same concentrations and quality of allosperm as in the laboratory is likely to be small.

3.4.4 Duration of the brooding cycle

The interval between the appearance of an oocyte in a particular female zooid and its transportation to the corresponding ovicell ranged from 1 to 8 d. Previously published residence times of visible oocytes inside the female coelom range from 1 to 4 d (Cancino, 1983, 1988; DJ Hughes, 1987). However, there is one important difference between the present findings and these previous studies. In the present study, observations were made on virgin colonies with a controlled allosperm dosage. Under these conditions a high proportion of the oocytes were found in female zooids whose ovicells had not yet been produced. These oocytes were then moved to the brood chamber only when the latter was nearing completion. The entire brooding cycle, from the moment the oocytes became visible inside the female coelom until they left the ovicell as a larva, ranged from 2 to 4 wk. However, these data only include oocytes present in female zooids whose ovicell was not yet occupied by an embryo. Under the more typical scenario when visible coelomic oocytes coincided with already occupied brood chambers (Experiment 1), the complete brooding cycle ranged from 5 to 7 weeks. Coelomic oocytes therefore are held back in the coelom until brooded larvae vacate the

ovicells. Differences in the duration of the complete brooding cycle between the first and second allosperm dosage (Experiment 2) could be interpreted as a consequence of more progeny being produced by females when their ovicells were occupied after the second allosperm dosage than when they were empty after the first dosage.

A possible explanation for shorter brooding cycles associated with smaller colonies (Experiment 3) is that more progeny were brooded in ovicells placed above active autozooids than in medium and larger colonies. Longer brooding period and low survivorship of embryos in brood chamber associated with unhealthy autozooids have been found in C. hyalina (personal observations).

Longer brooding period in colonies with greater cumulative reproductive activity (Experiment 6) could be interpreted as a consequence of female senescence. Female zooids in colonies with 4 months of reproductive activity had more brooding cycles (ca. 5-7 cycles) than colonies with shorter reproductive activity. This higher number of cycles is close to the maximum number described for C. hyalina under laboratory conditions (Cancino, 1983; DJ Hughes, 1987). It is possible that longer brooding period could be directly associated with female senescence. Although senescence may be avoided at the level of the genet (Jackson and Coates, 1986), in bryozoans senescence is frequently expressed at the level of the module or zooid (Ryland, 1979; Palumbi and Jackson, 1983; Muñoz et al., 1990; Bayer and Todd, 1997).

Female bias evidently does not influence brooding time and it seems, therefore the length of the brooding cycle is a more less constant value in C. hyalina. As reproductive success depends partially on female fecundity, the advantage of female bias clones in C. hyalina may lie in the production of more progeny rather than in a shortened brooding cycle.

3.4.5 Brooding success

In the laboratory-propagated colonies, proportion of embryos successfully leaving the ovicells as larvae was always high (ca. >80%). Similarly, brooding success was also high in wild colonies moved to the laboratory (ca. >70%). These values are similar to

those given by Cancino (1983) DJ Hughes (1987) and McCartney (1994). Once an embryo has passed from the female coelom to the brooding chamber its chances of completing the cycle are high. The only deleterious effect on the complete brooding cycle (i.e. from oocyte to larva) was found in big colonies (Experiment 3) and in colonies with greater cumulative reproductive activity (Experiment 6). In the first case, oocytes disappeared when the female zooids were associated with recycling autozooids (personal observation). In small and medium colonies recycling autozooids were also present but represented a small proportion of the colonies (personal observation). This suggests that a failure in fertilization or insufficient energetic support associated with females in unhealthy areas was responsible for the observed differences in brooding success. In the second case, the most aged females had experienced at least 7 brooding cycles before the experimental allosperm dosage took place (personal observations). This strongly suggests the existence of deleterious effects of previous brooding cycles (i.e. female ageing) on brooding success.

3.4.6 Settlement

Settlement was observed approximately from the third to ninth week in the laboratory propagated colonies. However, in the wild colonies settlement was observed from the first day after capture until approximately the fifth week. Absence of settlement before the third week in the laboratory-propagated colonies is in agreement with results of the appearance of the first and last oocytes. Since the laboratory-propagated colonies were virgin, settled larvae were not expected until the first batch of oocytes had completed the brooding cycle. Thus, the absence of settlement before the expected time confirms that progeny were fathered by the controlled allosperm dosage. On the other hand, cessation of settlement after the tenth week is in agreement with the appearance of the last oocytes inside the coelom. Considering the time the last oocytes were visible inside the coelom plus the average brooding time, further settlement was not expected beyond this time. Again for the wild colonies, last settlement observed in the third week is in agreement with the appearance of the last oocytes. This suggests that in the absence of self-fertilization, and knowing the duration of the brooding time, sperm storage could be assessed through observations on oocyte production or through observations on the temporal pattern of settlement.

3.4.7 Sperm movement

In Experiments 1-6 and 8, progeny were always produced in female zooids situated beyond the previous growing edge at the time of allosperm dosage. In the absence of self-fertilization and contamination of unwanted exogenous sperm, these result strongly suggests that once sperm have penetrated a colony they are transported from the point of entry to the site where the brooding will take place. The possibility that sperm could be expelled from a penetrated colony and then be captured in a different place should be rejected. Absence of oocytes, embryos and larvae in virgin colonies placed together with a clone-mate exposed previously to allosperm (Experiment 1), suggests that once sperm have penetrated a colony they do not leave the colony again. Progeny produced beyond the initial growing edge provide a visual representation of sperm movement inside colonies. Sperm should also be transported between areas within the original growing edges. This could be shown by the appearance of oocytes in female zooids not present at the moment that allosperm exposure took place. However, such appearance could also be explained by sperm captured and stored by zooids placed beneath the newly budded female zooids.

3.4.8 Sperm storage

The appearance of the last oocytes together with production of progeny by female zooids not present when the allosperm dosage took place (Experiments 1-6 and 8) demonstrate that long term sperm storage is possible in C. hyalina. The importance of sperm storage is strongly linked to an opportunity for sperm selection by females (i.e. cryptic female choice). Multiple mating is expected in colonies of C. hyalina growing in the field. However, progeny of colonies exposed to a cocktail of 3 different allosperm sources show that paternity occurred in the same proportions as the allosperm in the cocktail (Chapter 5). This suggests that allosperm storage has not evolved as a mechanism to enable female choice. However, sperm storage could have evolved as an adaptive response to assure a continuous supply for fertilization. This is particularly important in C. hyalina since self-fertilization is rare (this Chapter and all the others) and fertilization is dependent on the capture of diluted allosperm (see Chapter 2). Long term sperm storage in C. hyalina coupled with intra-colonial sperm movement enable colonies to control sperm usage and fertilization success. Relocation of stored sperm

from one place to another could enhance fertilization success by using female zooids associated with active feeding zooids. Low rates of abortion in areas with active autozooids support this hypothesis. This hypothesis also could explain the appearance of progeny beyond the original growing edge. In laboratory-reared colonies of C. hyalina the proportion of active zooids increases from the centre to the perimeter (personal observation). The same occurs in wild colonies of C. hyalina growing on their natural substratum (personal observation). This illustrates the advantage of relocating captured sperm to this peripheral zone, where newly budded zooids will soon become active, and free from any possible physiological load caused by previous polypide turnover associated with regression and regeneration cycles. Results of manipulation on autozooid regression and regeneration in C. hyalina (personal unpublished data) show that the polypide turnover time ranged from 1-3 d for zooids placed close to the growing edge (ca. 2-6 rows of zooids) and from 1 to 2 d in zooids close to the colonial centre (ca. 200 rows of zooids from the colonial edge). Moreover, the proportion of autozooids recovering from a manipulated recycling stage is close to 100% in autozooids close to the growing edge and from 3-40% in zooids close to the colonial centre (personal unpublished data). The active transfer of metabolites from the colony centre to the actively growing perimeter has been demonstrated in other bryozoans (Best and Thorpe, 1985; Miles et al., 1995). This again illustrates the adaptive benefit of relocation of captured allosperm to more vigorous regions of the receiver colony, in order to maximise fertilization success.

Production of progeny in small fragments of 10 and 3 active zooids (Experiment 8) strongly suggests that the presence of visible sexual zooids was not necessary for the capture, storage and use of allosperm. It is therefore possible that viable sperm are stored prior to fertilization in C. hyalina. Under natural conditions, colonies of C. hyalina could be receptive to allosperm and onset sexual activity as soon they can capture allosperm. Evidence of precocious insemination of oocytes in the ovary has been reported in other ovicellate brooders (Marcus, 1941; Corrêa, 1948; Dyrynda 1981a, Dyrynda and King, 1982). To date no information is available on C. hyalina sperm concentration in the field, however it is expected to be lower than the manipulated concentration used in the present study. Moreover, considering the effect of sperm concentration on fertilization success under laboratory conditions (see Appendix 2), it is

quite possible that colonies of C. hyalina could be facing sperm dilution limitations in the field. The consequences of early exposure to allosperm suspensions on the onset of sexual reproduction in the small colonial fragments and triple zooid colonies used in the Experiment S. are presented in the Chapter 7. In these colonies, the onset of sexual reproduction. apparent by production of female zooids and progeny, was earlier than in similar colonies maintained as controls under reproductive isolation. Previous work (Cancino, 1983) suggests the existence of a size threshold for sexual maturation, colonies becoming reproductive (male production) at a mean colony size of 57 autozooids (Cancino, 1983). However, observation on more than 400 small colonies growing on their natural substratum (see Appendix 3) suggests a smaller reproductive size threshold. Colonies as small as 20 zooids were found with only female sexual zooids. which even contained visible oocytes and embryos. Since in some of these colonies, the brood chambers were not yet completed but oocytes were visible inside the female coelom it maybe inferred that sperm were captured, stored and used for precocious fertilization. Precocious mating had been suggested as a possible mechanism of sperm storage in C. hyalina (Cancino, 1983). This seems to be supported by reports of precocious insemination of immature oocytes in bryozoans (Marcus, 1938; Dyrynda and King, 1982).

The importance of precocious insemination or becoming sexually receptive very early in ontogeny long before sexual maturity allows colonies to store sperm until needed. This could be interpreted as a mechanism evolved to trigger sexual reproduction as soon as sperm are available for collection without spending resources on production of brood chambers. Dense populations of C. hyalina growing in their natural substratum have been interpreted as a mean of minimising the chances of selfing (Ryland, 1976; Cancino et al., 1991). However, the rare occurrence of small colonies bearing reproductive zooids (see Appendix 3) could be interpreted as a consequence of the infrequent opportunity of such colonies to capture sperm under natural water regimes. This is supported by evidence from the consequences of water flow on fertilization success (see Chapter 4).

Expectation of survival of C. hyalina colonies occurring on their natural substrata, Fucus serratus and Laminaria saccharina, is at the most 4-6 months (Seed and O'Connor,

1981; Cancino, 1983). In such conditions, and without the existence of a trade-off between sexual reproduction and growth, colonies should reach sexual maturity when they are a few weeks old. Many long-lived clonal species delay reproduction throughout unfavourable conditions until reaching some minimum size (Harvell and Grosberg, 1988). However, in short-lived species such as C. hyalina, the onset of reproduction should begin as soon as intrinsic requirements have been achieved. Previous studies showed that reproduction in C. hyalina does not have a negative effect on somatic growth. suggesting that there is no advantage in delaying sexual reproduction (Cancino, 1983). However, differences in size between colonies exposed continually to allosperm suspensions and controls maintained under reproductive isolation (see Chapter 7) suggest that a negative effect of reproduction on somatic growth could indeed occur in C. hyalina. However, the concentration of sperm used in the continuous exposure experiments (see Chapter 7) must greatly exceed concentrations in the field. In the present study, storage of sperm and production of progeny by small non-reproductive colonies of C. hyalina support the idea of precocious insemination and sperm storage. Moreover, the capacity of sperm storage by non-reproductive or reproductive colonies could play an important role in the field, assuring that cross fertilization occurs even when the supply of allosperm is limited or intermittent. This is in agreement with experimental evidence of storage of exogenous sperm in Diplosoma listerianum (Bishop and Ryland, 1991) a species with a similar mating system to that in C. hyalina.

To date no information is available about the site and mechanism (i.e. prior to fertilization or post fertilization) of allosperm storage in C. hyalina. This information is crucial for understanding the physiological mechanism implicated in the maintenance of viable sperm in storage and the mechanism involved in intra-colonial transport. Sperm capture and entry in C. hyalina seems to be associated with protruded lophophores of active zooids (Marcus, 1938; see Chapter 4). If so, sperm should migrate from the coelom of the receptor autozooids to the coelom of female zooids. This idea has been supported by the occurrence of sperm in the funicular system that connects neighbouring zooids, and within male, female and autozooids (Marcus, 1938). These findings suggest that production of progeny by non-reproductive colonies taken from the field into reproductive isolation in the laboratory, described by Cancino (1983) could now be interpreted as sperm being stored before isolation.

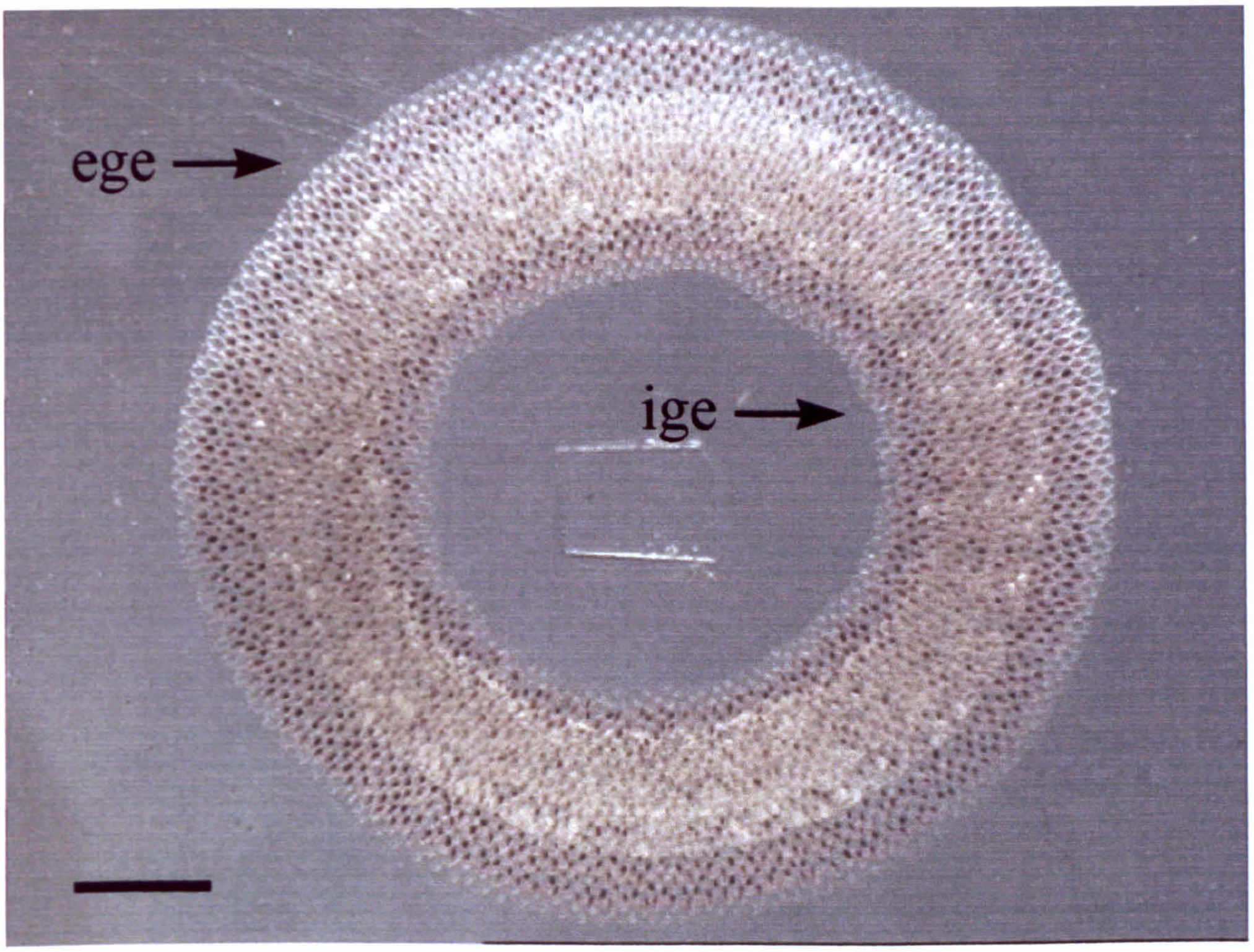

Plate 1: Celleporella hyalina: ring as used in the Experiment 4. (ege) external growing edge and (ige) internal growing edge. Scale bar = 4 mm.

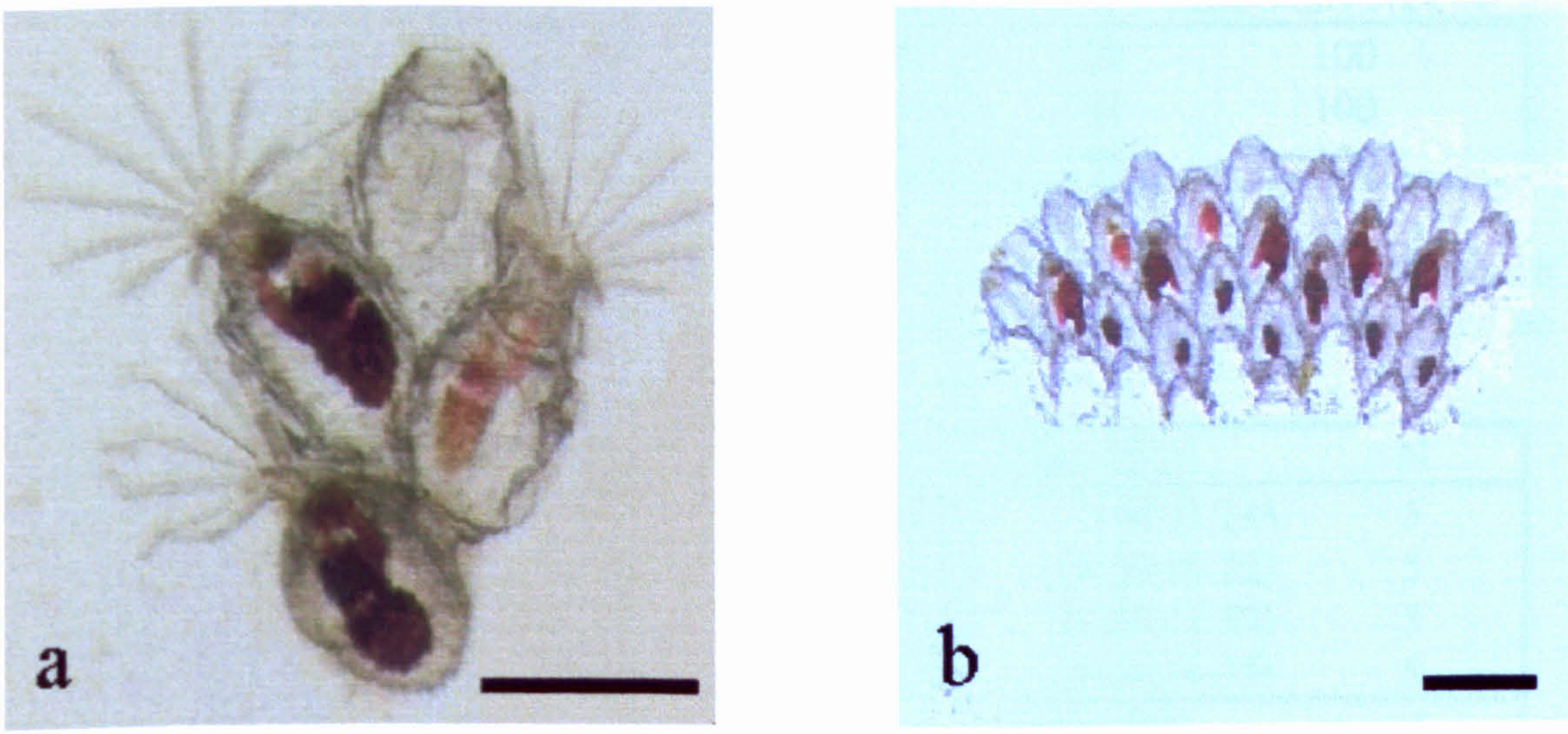

Plate 2: Celleporella hyalina: small colonies consisting of (a) 3 basal complete autozooids and (b) fragment of 10 basal complete autozooids (i.e.bearing a complete fuctional feeding apparatus), as used in the Experiment 8. Scale bars = 200 μm (a) and 300 μm (b).

Table 1: Experiment 1. Time (in days) of appearance of the first and last ovulated coelomic oocytes, and first and last settled larvae in colonies of C. hyalina after an allosperm dosage.

Trial 1:	range	mean (SD)	N
First oocyte	2-6	3.6 (1.1)	14
Last oocyte	19-35	26.6 (4.7)	14
First settled larvae	18-28	22.0 (3.4)	14
Last settled larvae	42-57	48.1 (4.3)	14
Trial 2:	range	mean (SD)	N
First oocyte	2-5	3.7 (1.2)	14
Last oocyte	19-34	29.6 (4.4)	14
First settled larvae	18-26	21.6 (2.9)	14
Last settled larvae	42-57	50.0 (5.4)	14

Table 2: Experiment 1. Duration of the brooding cycle (in days) and brooding success in colonies of C. hyalina. Brooding success was computed as (a) the proportion of oocytes successfully moving from the coelom to the ovicells (brooding success in the female body), (b) as the proportion of embryos successfully leaving the brood chamber as larvae (brooding success in the ovicells) and (c) as the proportion of initial oocytes successfully completing the full brooding cycle (complete brooding success). N = initial number of oocytes or embryos.

Trial 1:	range	mean (SD)	N	Brooding success
Days inside the female zooid	1-8	3.54 (1.57)	279	99.64
Days inside the ovicells	12-23	18.02 (2.77)	273	95.71
Total brooding time	14-28	21.56 (3.19)	273	97.90
Trial 2:	range	mean (SD)	N	Brooding success
Days inside the female zooid	1-7	2.85 (1.25)	280	100
Days inside the ovicells	12-22	17.40 (2.16)	280	100
Total brooding time	14-27	20.25 (2.63)	280	100

Table 3: Experiment 2. Time of appearance (in days) of the first and last ovulated coelomic oocytes, and first and last settled larvae in colonies of C. hyalina after an allosperm dosage.

First allosperm dosage:	range	mean (SD)	N
First oocyte	2-5	3.60 (1.14)	5
Last oocyte	17-31	24.60 (6.22)	5
First settled larvae	18-22	20.00 (1.58)	5
Last settled larvae	40-51	46.20 (4.55)	5
Second allosperm dosage:	range	mean (SD)	N
First oocyte	1-3	2.20 (0.84)	5
Last oocyte	20-30	23.20 (4.09)	5
First settled larvae	19-23	21.80 (1.64)	5
Last settled larvae	41-48	44.20 (2.59)	5

Table 4: Experiment 2. Legend as for Table 2.

First allosperm dosage:	range	mean (SD)	N	brooding success
Days inside the female zooid	1-6	3.07 (1.23)	100	99.00
Days inside the ovicells	12-21	16.78 (2.03)	98	99.00
Total brooding time	18-28	19.87 (2.38)	98	99.00
Second allosperm dosage:	**range**	**mean (SD)**	**N**	**brooding success**
Days inside the female zooid	1-4	2.15 (0.85)	100	100
Days inside the ovicells	12-22	16.83 (2.12)	100	100
Total brooding time	14-23	20.79 (2.91)	100	100

Table 5: Experiment 3. Time of appearance (in days) of the first and last ovulated coelomic oocytes, and first and last settled larvae in colonies of C. hyalina after an allosperm dosage.

Small size colonies:	range	mean (SD)	N
First oocyte	3-4	3.3 (0.6)	3
Last oocyte	18-30	23.0 (6.4)	3
First settled larvae	17-23	20.3 (3.1)	3
Last settled larvae	42-53	46.3 (5.9)	3
Medium size colonies:	**range**	**mean (SD)**	**N**
First oocyte	2-3	2.7 (0.6)	3
Last oocyte	19-31	26.3 (6.4)	3
First settled larvae	19-20	19.67 (0.6)	3
Last settled larvae	43-56	48.33 (6.8)	3
Large size colonies:	**range**	**mean (SD)**	**N**
First oocyte	2-3	2.7 (0.6)	3
Last oocyte	19-29	25.0 (5.3)	3
First settled larvae	19-22	20.3 (1.5)	3
Last settled larvae	43-56	47.3 (7.5)	3

Table 6: Experiment 3. Legend as for Table 2.

Small size colonies:	range	mean (SD)	N	brooding success
Days inside the female zooids	1-5	2.90 (0.97)	60	100
Days inside the ovicells	12-20	15.78 (1.75)	60	100
Total brooding time	15-24	18.68 (2.29)	60	100
Medium size colonies:	**range**	**mean (SD)**	**N**	**brooding success**
Days inside the female zooid	1-6	2.81 (0.97)	60	98.33
Days inside the ovicells	12-21	15.83 (2.15)	58	98.25
Total brooding time	14-25	18.64 (2.31)	57	96.67
Large size colonies:	**range**	**mean (SD)**	**N**	**brooding success**
Days inside the female zooid	1-4	2.58 (0.83)	60	91.67
Days inside the ovicells	13-22	17.15 (2.22)	54	98.25
Total brooding time	15-26	19.72 (2.41)	53	88.33

Table 7: Experiment 4. Time of appearance (in days) of the first and last ovulated coelomic oocytes, and first and last settled larvae in colonies of C. hyalina after an allosperm dosage.

Trial 1:	range	mean (SD)	N
First oocyte	2-4	3.0 (2.0)	3
Last oocyte	24-32	27.7 (4.0)	3
First settled larvae	20-23	34.2 (1.5)	3
Last settled larvae	42-53	46.7 (5.7)	3
Trial 2:	range	mean (SD)	N
First oocyte	2-3	2.3 (2.0)	3
Last oocyte	24-31	28.00 (2.0)	3
First settled larvae	18-26	34.2(1.5)	3
Last settled larvae	42-49	44.7 (3.8)	3

Table 8: Experiment 4. Legend as for Table 2.

Trial 1:	range	mean (SD)	N	brooding success
Days inside the female zooid	1-5	2.81 (0.76)	60	96.67
Days inside the ovicells	12-19	16.12 (1.91)	58	100
Total brooding time	15-23	18.93 (1.99)	58	96.67
Trial 2:	range	mean (SD)	N	brooding success
Days inside the female zooid	1-5	2.75 (0.79)	60	100
Days inside the ovicells	12-20	16.18 (1.65)	60	100
Total brooding time	15-22	22.47 (2.36)	60	100

Table 9: Experiment 5. Time of appearance (in days) of the first and last ovulated coelomic oocytes, and first and last settled larvae in colonies of C. hyalina after an allosperm dosage.

Female bias clones:	range	mean (SD)	N
First oocyte	2-4	2.8 (0.7)	9
Last oocyte	25-36	29.3 (4.0)	9
First settled larvae	19-27	23.67 (2.6)	9
Last settled larvae	38-52	44.6 (4.1)	9
Normal clones:	range	mean (SD)	N
First oocyte	2-4	2.67 (0.7)	9
Last oocyte	24-33	29.1 (3.1)	9
First settled larvae	19-27	22.1 (2.4)	9
Last settled larvae	43-52	50.0 (3.0)	9

Table 10: Experiment 5. Legend as for Table 2.

Female bias colonies:	range	mean (SD)	N	brooding success
Days inside the female zooid	2-4	2.70 (0.67)	60	100
Days inside the ovicells	12-22	15.98 (2.93)	60	100
Total brooding time	15-25	18.68 (2.30)	60	100
Normal clones:	range	mean (SD)	N	brooding success
Days inside the female zooid	2-5	3.00 (0.86)	60	100
Days inside the ovicells	12-21	15.45 (1.93)	60	100
Total brooding time	14-24	18.45 (2.17)	60	100

Table 11: Experiment 6. Time of appearance (in days) of the first and last ovulated coelomic oocytes, and first and last settled larvae in colonies of C. hvalina after an allosperm dosage.

One month aged colonies:	range	mean (SD)	N
First oocyte	3-4	3.33 (0.6)	3
Last oocyte	25-31	29.0 (3.5)	3
First settled larvae	20-24	22.0 (2.0)	3
Last settled larvae	35-50	47.7 (2.5)	3
Two months aged colonies:	range	mean (SD)	N
First oocyte	2-4	3.00 (1.0)	3
Last oocyte	22-35	26.7 (7.2)	3
First settled larvae	25-26	25.3 (0.6)	3
Last settled larvae	44-50	46.7 (3.1)	3
Four months aged colonies:	range	mean (SD)	N
First oocyte	2-3	2.7 (0.6)	3
Last oocyte	26-31	28.0 (2.7)	3
First settled larvae	22-25	23.7 (1.5)	3
Last settled larvae	45-53	48.3 (4.1)	2

Table 12: Experiment 6. Legend as for Table 2.

One month aged colonies:	range	mean (SD)	N	brooding success
Days inside the female zooid	2-5	2.85 (0.67)	90	97.78
Days inside the ovicells	12-22	15.76 (2.05)	88	100
Total brooding time	14-26	18.61 (2.14)	88	97.78
Two-months aged colonies:	range	mean (SD)	N	brooding success
Days inside the female zooid	1-5	2.85 (0.81)	90	96.67
Days inside the ovicells	12-21	15.63 (2.18)	87	100
Total brooding time	14-25	18.48 (2.35)	87	96.67
Four-months aged colonies:	range	mean (SD)	N	brooding success
Days inside the female zooid	2-5	2.86 (0.74)	90	85.56
Days inside the ovicells	12-24	17.34 (2.67)	77	84.68
Total brooding time	15-26	20.23 (2.66)	65	72.22

Table 13: Experiment 7. Time of appearance (in days) of the first and last ovulated coelomic oocytes. and first and last settled larvae in colonies of C. hyalina collected from the field.

1998:	event	range	mean (SD)	N (colonies)
May - Beaumaris:				
	Last oocyte	4-15	8.83 (3.50)	12
	Last settled larvae	25-36	30.92 (3.37)	12
June - Beaumaris:				
	Last oocyte	6-17	11.08 (3.87)	12
	Last settled larvae	16-35	26.08 (6.01)	12
May - C. Island				
	Last oocyte	3-15	6.50 (3.68)	12
	Last settled larvae	17-34	22.25 (4.35)	12
June - C. Island:				
	Last oocyte	4-15	9.42 (3.46)	12
	Last settled larvae	16-27	21.33 (3.58)	12
1999:				
May - Beaumaris:				
	Last oocyte	3-13	8.83 (3.46)	12
	Last settled larvae	21-33	28.58 (4.25)	12
June - Beaumaris:				
	Last oocyte	2-23	13.08 (7.25)	12
	Last settled larvae	19-37	28.17 (7.17)	12
May - C. Island				
	Last oocyte	3-14	6.25 (3.14)	12
	Last settled larvae	16-27	21.33 (3.58)	12
June - C. Island:				
	Last oocyte	9-15	11.75 (1.96)	12
	Last settled larvae	21-31	26.25 (3.33)	12

Table 14: Experiment 7. Duration of the brooding cycle (in days) and brooding success in colonies of C. hyalina moved from the field to reproductive isolation in the laboratory. Legend as for Table 2.

Beaumaris:	range	Mean (SD)	N	brooding success
Days inside the female zooid	1-5	2.55 (0.96)	60	81.67
Days inside the ovicells	13-19	16.06 (1.47)	46	86.49
Total brooding time	15-24	18.57 (1.98)	46	76.67
Church Island:	range	mean (SD)	N	brooding success
Days inside the female zooid	1-4	2.45 (0.76)	60	93.33
Days inside the ovicells	12-21	15.94 (1.67)	52	92.72
Total brooding time	15-24	18.36 (1.85)	52	86.67

Table 15: Experiment 8, Treatment 1; parental fingerprint score for progeny produced by triple zooid colonies of C. hyalina. Colonies were exposed to a mixture of 3 sperm sources (A_1, M_1, E_1) for 2 h during the first day and then maintained under reproductive isolation for 10 wk. In the last column, the first values correspond to number of embryos produced per colony, and the second values correspond to number of progeny used in the scoring.

First experimental run	Donor clones fathering the progeny			Total progeny (produced-scored)
Parental clones:	A_1	M_1	E_1	
A_1 x M_1	0	0	1	1-0
A_1 x E_1	-	-	-	0
Q_1 x D_1	0	1	0	1-0
Q_1 x J_1	1	1	0	2-0
Second experimental run	**Donor clones fathering the progeny**			**Total progeny (produced-scored)**
Parental clones:	A_1	M_1	E_1	
A_1 x M_1	0	0	2	2-2
A_1 x E_1	0	2	0	2-2
Q_1 x D_1	-	-	-	0
Q_1 x J_1	1	1	0	2-2

Table 16: Experiment 8, Treatment 2; Parental fingerprint score for progeny produced by triple zooid colonies of C. hyalina. Colonies were exposed to a mixture of 3 sperm sources A_1, M_1, E_1) for 2 h during the first day and then exposed continuously to allosperm from clone N_2 for 10 wk. In the last column, the first values correspond to number of embryos produced per colony, and the second values correspond to number of progeny used in the scoring.

Second experimental run	Donor clones fathering the progeny				Total progeny sampled-scored
Parental clones of the receptor colonies:	A_1	M_1	E_1	N_2	
A1 x M1	1	0	2	7	10-10
A1 x E1	0	1	1	8	10-10
Q1 x D1	2	1	0	7	10-10
Q1 x J1	3	1	0	6	10-10

Table 17: Experiment 8. Treatment 1,.Parental fingerprint score for progeny produced by 10-zooids colonies of C. hvalina. Colonies were exposed to a mixture of 3 sperm sources (A_1, M_1, E_1) for 2 h during the first day and then maintained under reproductive isolation for 10 wk. In the last column, the first values correspond to number of embryos produced per colony, and the second values correspond to number of progeny used in the scoring.

First experimental run	Donor clones fathering the progeny			Total progeny (produced-sampled)
Receptor colonies:	A_1	M_1	E_1	
A_1	0	5	0	5-5
D_1	3	1	0	4-4
E_1	0	2	0	2-2
J_1	2	2	0	4-4
M_1	0	0	0	0
Q_1	2	2	0	4-4
Second experimental run	**Donor clones fathering the progeny**			**Total progeny (produced-sampled)**
Receptor clones:	A_1	M_1	E_1	
A_1	0	1	0	1-1
D_1	0	1	1	2-2
E_1	0	0	0	0
J_1	0	2	0	3-2
M_1	1	0	0	1-1
Q_1	0	0	0	0-0

Table 18: Experiment 8. Fingerprint score for progeny produced by 10-zooids colonies of C. hyalina. Colonies were exposed to a mixture of 3 sperm sources A_1, M_1, E_1) for 2 h during the first day and then exposed continuously to allosperm from clone N_2 for 10 wk. (*=one sample did not work; ** extra samples were taken). In the last column, legend as for Table 17.

First experimental run	Donor clones fathering the progeny				Total progeny (sampled-scored)
Receptor clones:	A_1	M_1	E_1	N_2	
A_1	0	1	0	9	10-10
D_1	0	2	1	7	10-10
E_1	2	0	1	7	10-10
J_1	2	1	0	15	18-18**
M_1	2	0	0	8	10
Q_1	0	4	1	18	25-23**
Second experimental run	Donor clones fathering the progeny				Total progeny (sampled-scored)
Receptor clones:	A_1	M_1	E_1	N_2	
A_1	1	0	0	12	13-13
D_1	1	0	0	4	5-5
E_1	2	0	0	2	5-5*
J_1	0	0	0	10	10-10**
M_1	1	0	0	4	5-5
Q_1	1	0	0	4	5-5

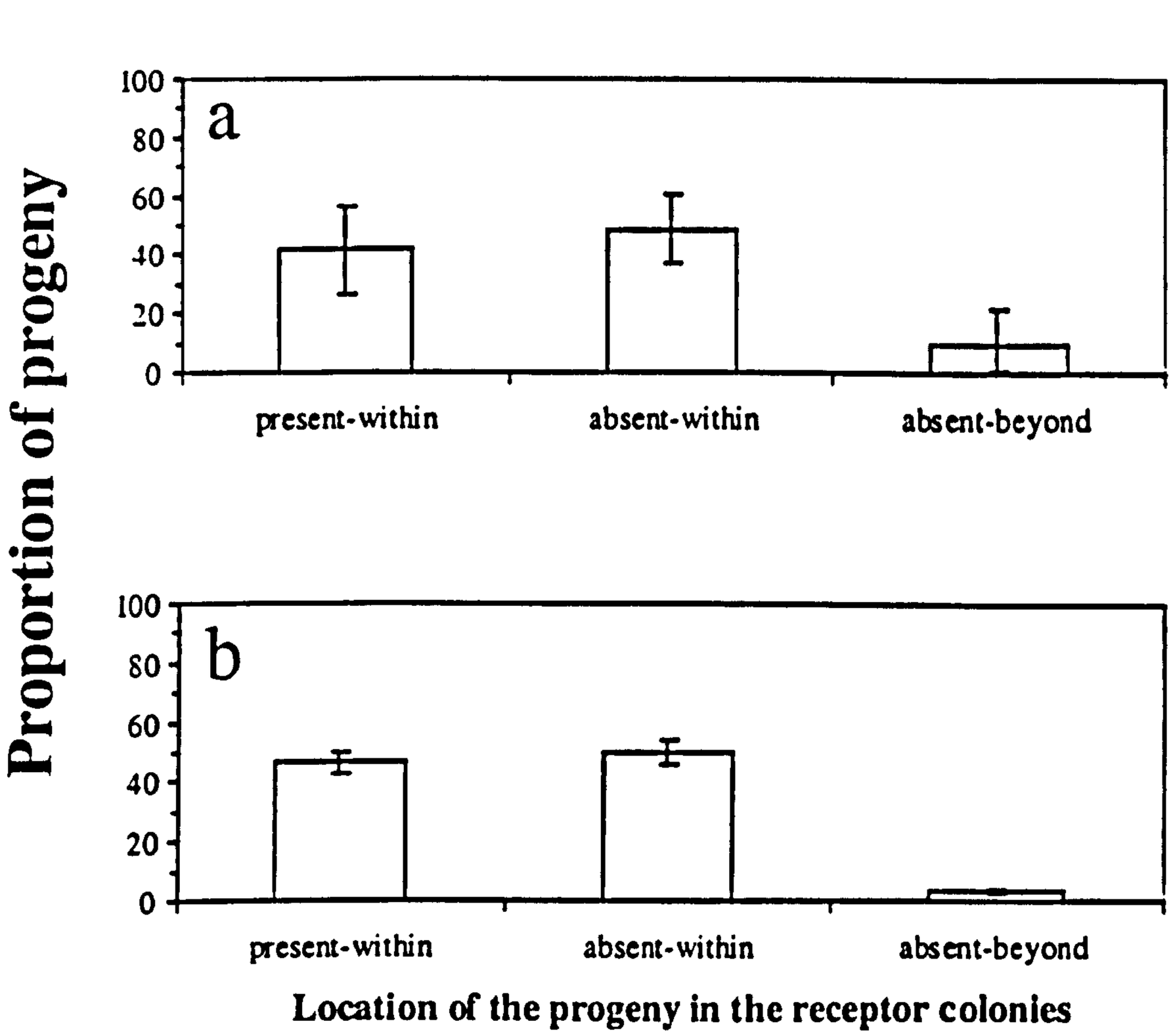

Figure 1: Experiment 1. Intra-colonial position of progeny within colonies of C. hyalina. Proportion of progeny is the number of embryos that were found in a particular location within the receptor colonies out of the total number of embryos produced per colony. Intra-colonial locations are: female zooids present within the colonial growing edge at the moment that allosperm dosage took place (present-within); female zooids absent within the colonial growing edge at the moment that allosperm dosage took place (absent-within) and female zooids budded beyond this growing edge (absent-beyond). Graph 1a corresponds to the first run and graph 1b to the second one. Data are mean and SD.

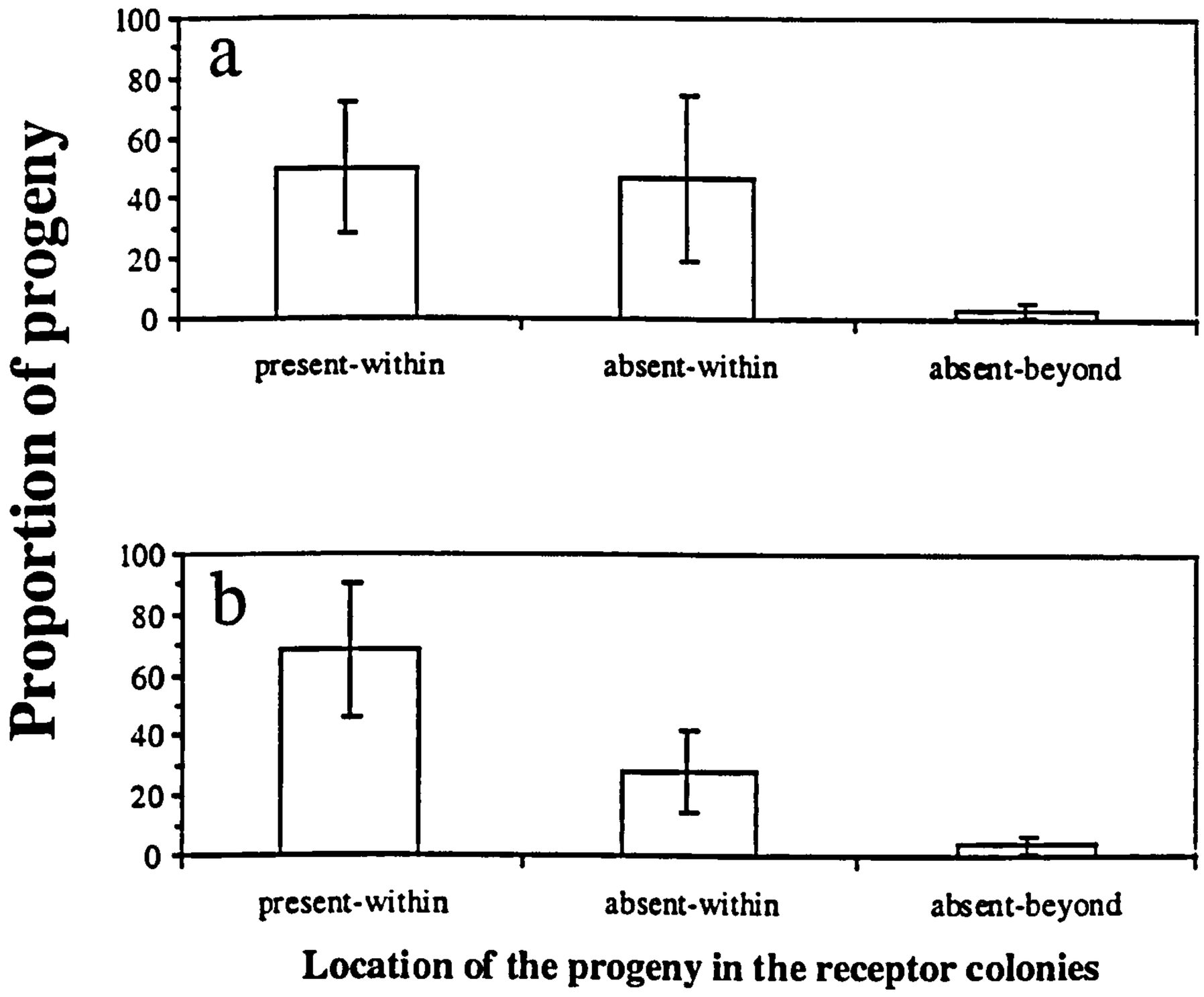

Figure 2: Experiment 2. Effect of timing of allosperm dosage on intra-colonial position of progeny within colonies of C. hyalina. Proportion of progeny is the number of embryos that were found in a particular location within the receptor colonies out of the total number of embryos produced per colony. Intra-colonial locations are: female zooids present within the colonial growing edge at the moment that allosperm dosage took place (present-within); female zooids absent within the colonial growing edge at the moment that allosperm dosage took place (absents-within) and female zooids budded beyond this growing edge (absent-beyond). Graph 3a show the results after the first allosperm dosage, and graph 3b show the results after the second allosperm dosage. Data are mean and SD.

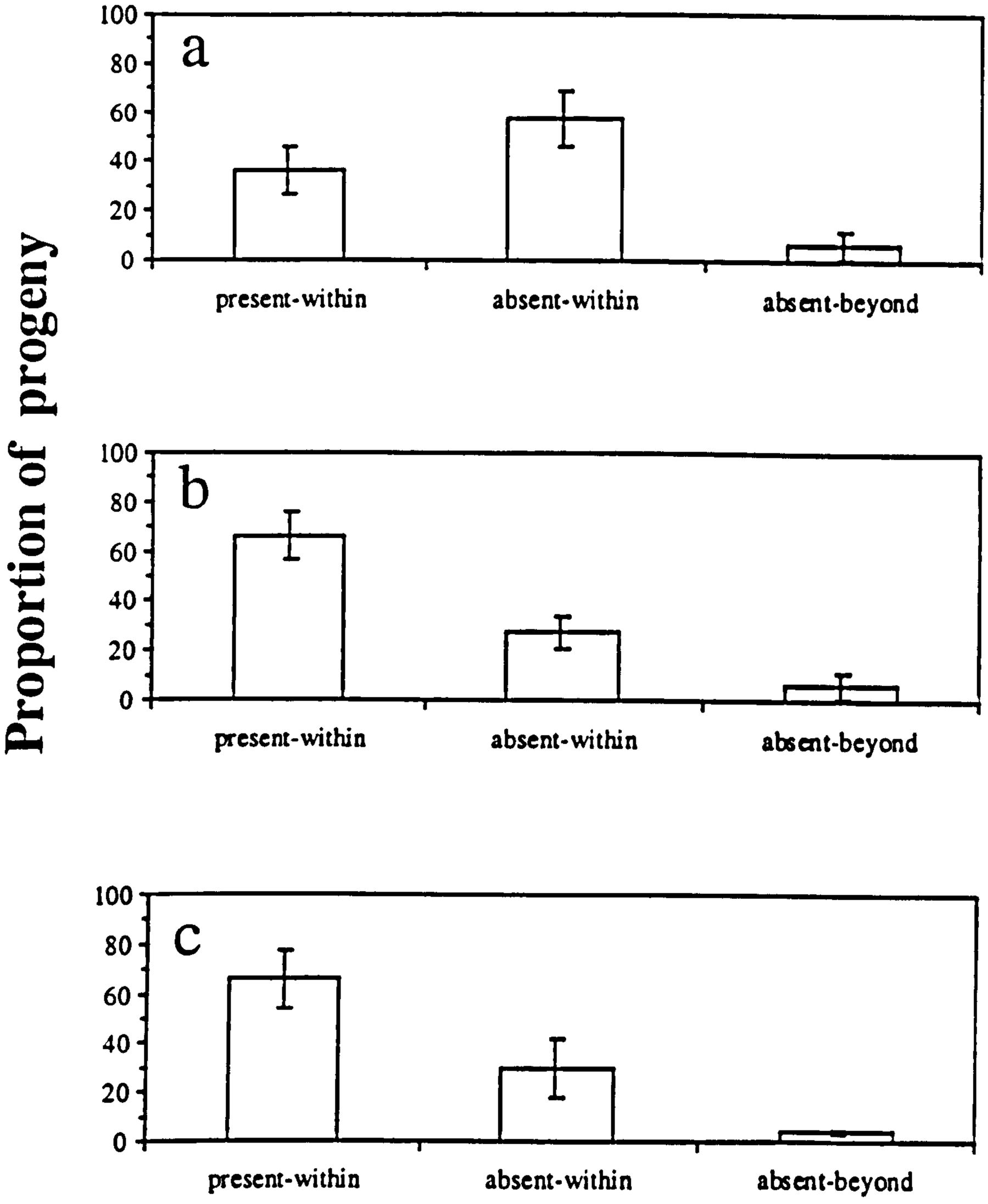

Figure 3: Experiment 3. Effect of colonial size on position of progeny within colonies of C. hyalina. Proportion of progeny is the number of embryos that were found in a particular location within the receptor colonies out of the total number of embryos produced per colony. Intra-colonial locations are: female zooids present within the colonial growing edge at the moment that allosperm dosage took place (presents-within); female zooids absent within the colonial growing edge at the moment that allosperm dosage took place (absents-within) and female zooids budded beyond this growing edge (absents-beyond). Graphs (a), (b) and (c) show results from small colonies, medium and large colonies respectively. Data are mean and SD.

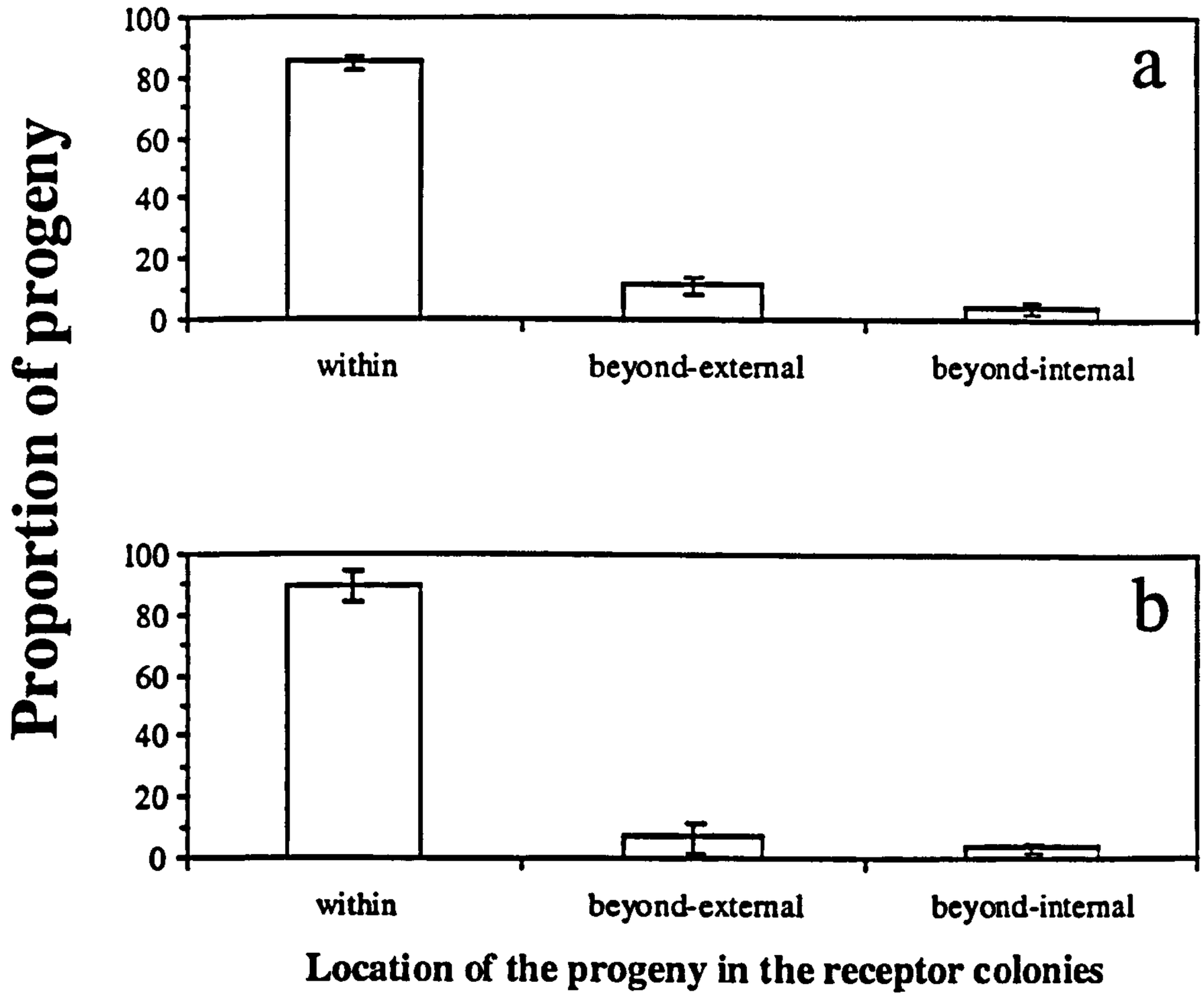

Figure 4: Experiment 4. Intra-colonial position of progeny within colonies of C. hyalina. Proportion of progeny is the number of embryos that were found in a particular location within the receptor colonies out of the total number of embryos produced per colony. Intra-colonial location are: embryos brooded within the colonial growing edge at the moment that allosperm dosage took place (within); embryos brooded in female zooids placed beyond the external growing edge (beyond-external) and embryos budded in female zooids placed beyond the internal growing edge (beyond-internal). Graph 4a corresponds to the first run and graph 4b to the second one. Data are mean and SD.

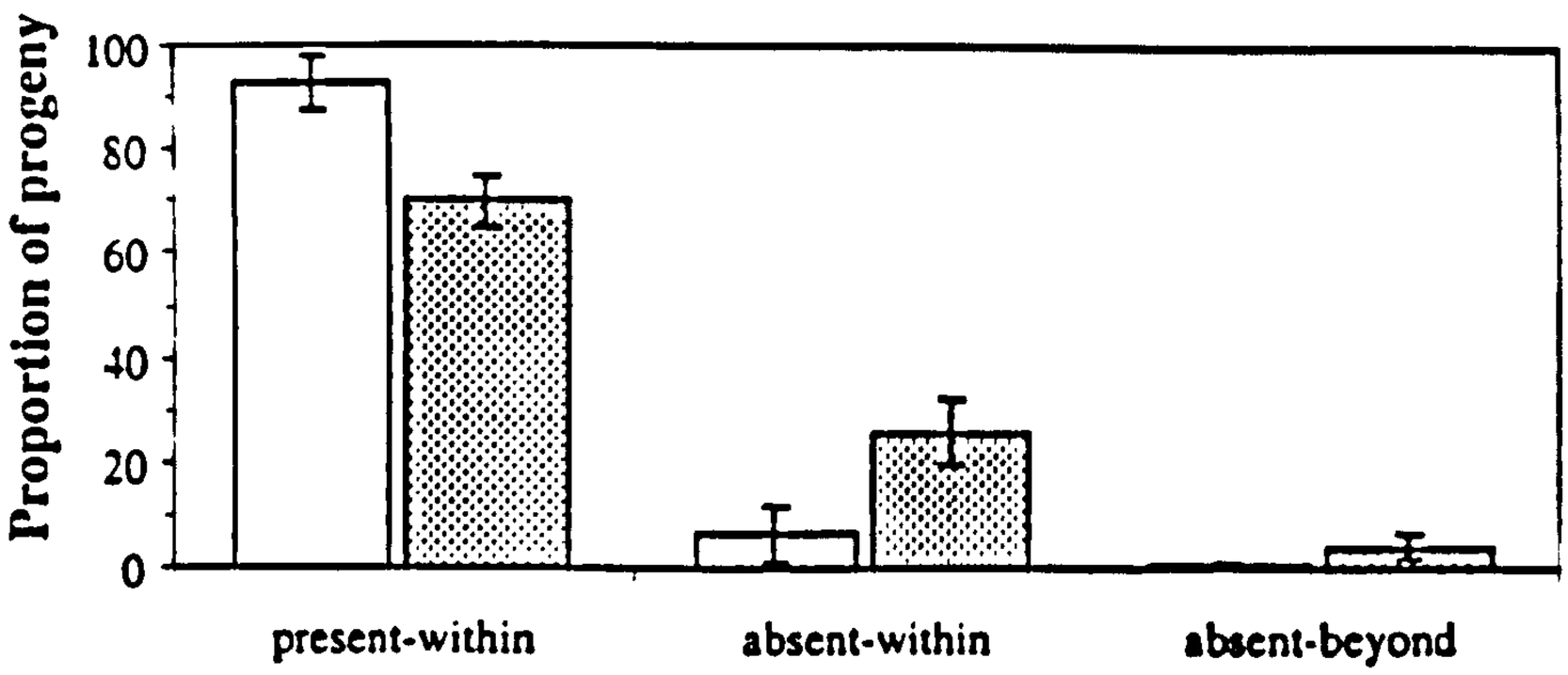

Figure 5: Experiment 5. Effect of female investment on intra-colonial position of progeny within colonies of C. hyalina. Proportion of progeny is the number of embryos that were found in a particular location within the receptor colonies out of the total number of embryos sired per colony. Intra-colonial locations are: embryos brooded within the colonial growing edge at the moment that allosperm dosage took place (present-within); embryos brooded in female zooids placed beyond the external growing edge (absent-within) and embryos budded in female zooids placed beyond the internal growing edge (absent-beyond). White bars are data from 3 colonies of 3 female bias clones and shaded bars are data from 3 colonies of 3 normal clones. Data are mean and SD.

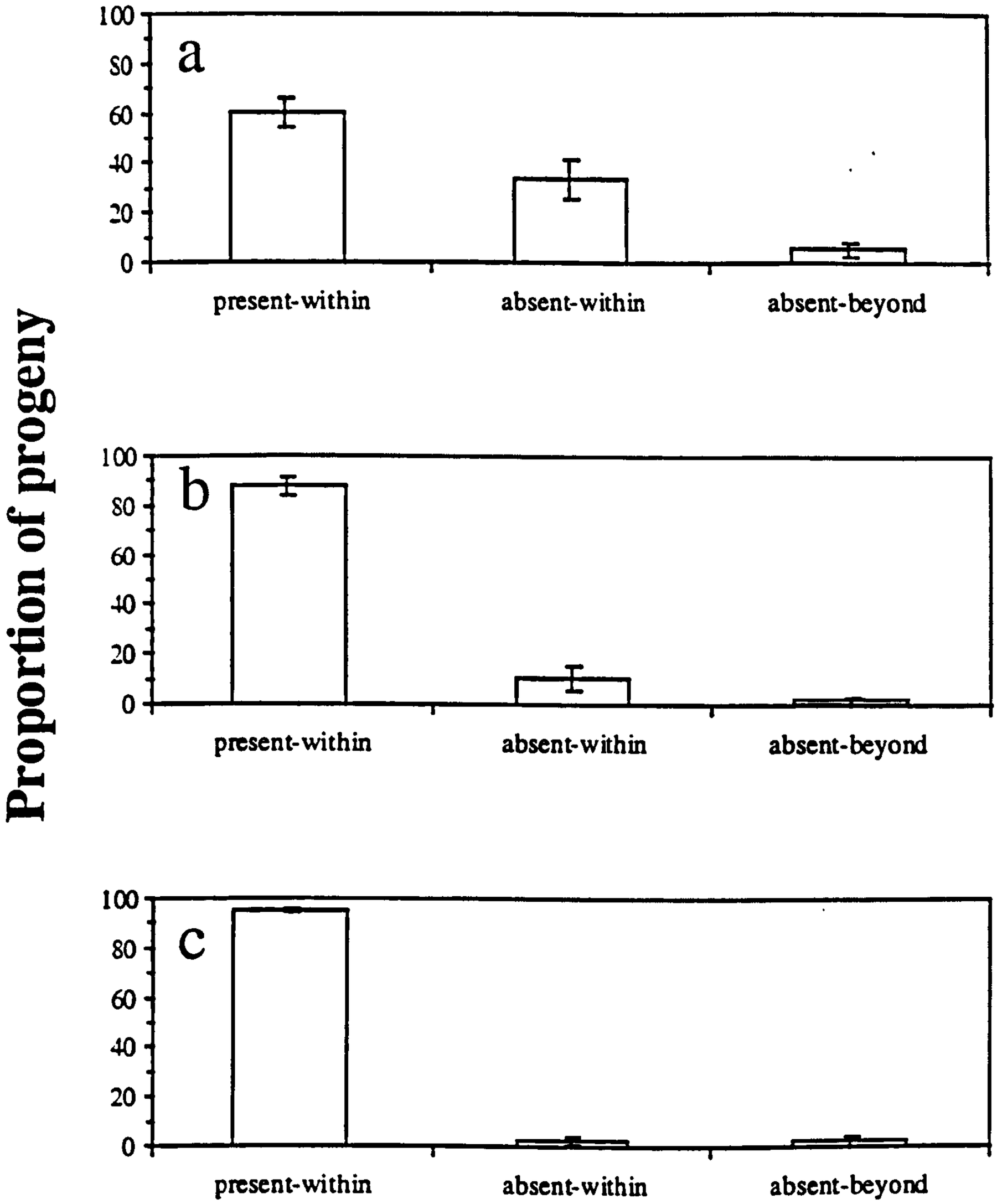

Figure 6: Experiment 6, effect of female ageing on intra-colonial position of progeny in C. hyalina. Proportion of progeny is the number of embryos that were found in a particular location within the receptor colonies out of the total number of embryos produced per colony. Intra-colonial locations are: embryos brooded within the colonial growing edge at the moment that allosperm dosage took place (present-within); embryos brooded in female zooids placed beyond the external growing edge (absent-within) and embryos budded in female zooids placed beyond the internal growing edge (absent-beyond). Graph (a), (b) and (c) are data from colonies with 1, 2 and 4 months of cumulative reproductive activity respectively. Data are mean and SD.

Chapter 4:
The effect of feeding activity and water flow on sperm release and fertilization success.

4.1 Introduction

In marine filter feeding organisms with ent-aquasperm (sensu Rouse and Jamienson, 1987), sperm are released into the ambient water and drawn in by the inhalant feeding current of the females, fertilization of retained eggs occurs internally. Internal fertilization in Bryozoa and Ascidiacea, two taxa of colonial hermaphroditic invertebrates, has been reviewed by Ryland and Bishop (1993). They note that although there is some evidence of self-fertilization for bryozoans (Hunter and Hughes, 1993c), direct observations of both sperm release and sperm uptake suggest that cross-fertilization is possible (Silén, 1966, 1972; Temkin, 1994, 1996). The effect of water flow on invertebrate filter feeding is well documented (Okamura, 1984, 1985; Petterson, 1984; Cancino and Hughes, 1987; Sebens et al., 1991; Okamura and Partridge, 1999). Bryozoans are active filter feeding organisms in which protruded lophophores of autozooids remove food particles from the seawater (Bullivant, 1968). The mechanism involved in food capture and the role played by protruded lophophores are described by Riisgård and Manríquez (1997). Published information suggests that many colonial attributes can be influenced by flow velocity around the colonies (Cancino & Hughes, 1987; Okamura, 1985, 1999; DJ. Hughes 1989, 1992; Muñoz 1996). Despite the absence of a clear effect of water flow on sexual allocation reported by Cancino and Hughes (1987), water flow may still be linked to sperm capture and fertilization success in filter feeding organisms with internal fertilization. Studies on D. listerianum suggest that cross-fertilization is not dependent on the interception of dense clouds of gametes (Bishop, 1998). However, observations on hydroids suggest that suspension feeding could increase chances of fertilization by utilizing endogenous water flow (Miller, 1994). This could play an important role if allosperm are the main or only source of compatible sperm for marine invertebrates with internal fertilization. Colonies of the gymnolaemate bryozoan Celleporella hyalina (L.) are common epialgal suspension-feeders with internal fertilization. To date, male lophophore protrusion has been only occasionally or never observed (Marcus, 1938; Cancino, 1983; Hughes, 1987). Preliminary observations have demonstrated sperm release following protrusion of the

male lophophore (see Appendix 2). For cheilostomes, it has been proposed that sperm enter the fertile zooids through the supraneural coelomopore (Silén, 1966), which in C. hyalina is associated with basal autozooids (Marcus, 1938). It has been suggested however, that the presence of a supraneural coelomopore in female zooids of C. hyalina could play a role in sperm entry (DJ Hughes, 1987). Moreover, the aperture of the operculum associated with ascus musculature in the female zooids of C. hyalina also has been suggested as a route of entrance of sperm to the maternal coelom (Ostrovsky, 1998). To date, however, the route for sperm entry into receptor colonies remains largely unknown. In C. hyalina the first ovulated coelomic oocytes (hereafter oocytes) are detectable in the female coelom within a few weeks following exposure to sexually compatible allosperm suspensions (see Chapter 3). Since self-fertilization is rare in the Welsh C. hyalina (Cancino, 1983; Hunter 1993a; Hunter and Hughes 1993c; Hoare et al., 1999; see Chapter 7), this allows the presence of oocytes in virgin receptor colonies to be used as a measure of allosperm utilization under different water flow conditions. Moreover, the possibility of experimentally inducing sperm release (see Appendix 2) enables fresh suspensions of allosperm to be used, so avoiding risk associated with the use of aged sperm (see Chapter 2). In the present study, two aspects of the reproductive biology of C. hyalina were investigated: sperm release and fertilization success in connection with water flow. The potential role played by the protruded lophophore in association with the capture and entry of allosperm was also studied. Such information is important for understanding the dynamics of fertilization in sessile organisms with ent-aquasperm.

4.2 Materials and methods

Experimental clones were originated, propagated and maintained in reproductive isolation using the standard protocols (see Appendix 1). The effect of water regime on sperm release and fertilization success was examined using a small-scale unidirectional 2 l flume (20 cm length, 1.5 cm width). Water velocities in the flume were controlled by a calibrated valve connected to an electric pump. In order to minimize turbulence, the water inlet was passed through a block of parallel plastic straws (ca.1 mm in diameter) placed at the entrance of the flume. Water velocities in the flume were determined by following trajectories of R. reticulata at a concentration of 100 cells·µl^{-1} using a video camera (Kappa CF 11/1) connected to a horizontally placed inverted microscope (Labovert FS), and a video recorder (Panasonic NV FS200 HQ) capturing 50 half frames per second. The velocity of cells passing about 50-100 µm over the top of the protruded lophophores was measured. To do this, trajectories were traced in an acetate sheet mounted onto the video screen, where the positions of R. reticulata were marked at 0.02 s time intervals. Four water flow conditions were used: still water; low flow (1-2 cm·s^{-1}); medium flow (6-7 cm·s^{-1}) and high flow (15-16 cm·s^{-1}). Adverse mechanical effects of the electric pump on the capacity of sperm to produce progeny in receptor colonies were not found in preliminary experiments. The number of basal males, frontal males and total number of both active and recycling autozooids were counted in all the colonies used for studying sperm release. When fertilization success was studied, the number of female zooids and both active and recycling autozooids were noted at the beginning of the experimental period. Because of the small size of the working section in the flume all experimental colonies used in this apparatus were colonies of 0.9 to 1.1 cm in diameter (ca. 400 to 720 basal zooids). However, when the flume was not used, experimental colonies were between 2.0 to 3.0 cm in diameter (ca. 2400 to 5400 basal zooids). All the experiments were conducted in a controlled temperature room with a 12:12 dark/light photoperiod. Experiments using the flume were conducted on a laboratory bench at room temperature, but the water was maintained at 16-17°C by placing the flume over a metallic cooling plate connected to a water bath. When allosperm suspensions were required a light/dark shift technique was applied in colonies maintained in darkness overnight (see Appendix 2). Experimental colonies were fed on a daily basis using R. reticulata in order to achieve an approximate final concentration of

10-20 cells·µl^{-1} as recommended by Riisgård and Manríquez (1997). Water changes were conducted every second day and the colonies cleaned once a week. Before analysis, data set was inspected for non-normality and homoscedasticity. If assumptions of homogeneity of variance and normality were not rejected, statistical treatment included one way and simple factorial two-way analysis of variance (ANOVA). Lack of normality and homoscedasticity in raw data sets were corrected by appropriate transformation. Thus, ratio and percentage data were transformed to square root and arcsine values respectively whereas, logarithmic transformations were used in other cases (Sokal and Rolf, 1995). When normality and equality of variance in the data set was not achieved by transformations, the nonparametric Kruskal-Wallis test was applied. All the statistical analyses were conducted using SPSS 8.0 data analysis program. In all the figures (see results), variation around the mean is given as one standard error (SE).

4.2.1 Sperm release

4.2.1.1 Experiment 1: the effect of water flow on sperm release

The effect of water flow on sperm release was examined by placing donor colonies in the bottom of the flume 10 cm from the water inlet. A small piece of double-sided adhesive tape was used to attach the acetate sheet bearing the donor colony in place. Colonies were maintained for 24 h in darkness and then cleaned with a 10 min rinse of FSW and placed in the flume. Once the donor colony was placed in the flume, 2 l of FSW were carefully poured from a beaker into the flume and then the required water flow generated. The light was switched on and the colony maintained in the flume for 1 hour. After this period of time the colonies were removed and the water in the flume placed and stirred in a 5 l beaker. Samples of 30 ml were taken and sperm counted (see Appendix 2). Ramets of 5 different clones were studied in different experiments, one ramet from each being assigned to each experimental treatment: still water, low, medium and high water flow conditions. Manipulations in darkness were made using a dim, red torch light. As just one flume was available, it was not possible to run all the treatments at the same time. Sperm release was expressed as number of sperm per ml per active male per colony.

4.2.2.2 Experiment 2: the effect of water flow in male lophophore protrusion

The effect of water flow on male lophophore protrusion was investigated using the same flume and water flow conditions described above. All the experimental colonies were maintained for 24 h in darkness before being exposed to light in the corresponding water flow condition. After the light was switched on, male lophophore protrusion was assessed by continuous observations for 30 minutes using a stereo microscope (Wild M3) mounted above the flume was used to assess the proportion of protruded male lophophores. Observations were made on a section of 6.25 mm^2 (ca. 45 basal zooids) of the experimental colonies. At each experimental water flow condition, one ramet from each of 4 different clones was used. Male protrusion was expressed as the number of protrusions per male per minute per colony.

4.2.2.3 Experiment 3: the effect of lophophore protrusion on sperm release

In order to investigate if total male lophophore protrusion is required for sperm release, the following experiment was designed. Two ramets of each of 4 clones with male zooids bearing visibly active sperm in their coelom were used. The colonies were maintained in darkness for 24 h and then cleaned with 10 min rinses of FSW before being assigned randomly to the experimental treatment in a 250 ml glass vessel. As in the previous experiments, dim red light was used during this procedure. One ramet from each clone was covered with a soft 2 mm thick sheet of expanded polyethylene used for packing (Treatment 1), using plastic clips to maintain the sheet in position. The second ramet was maintained without any impediment for lophophore protrusion (Treatment 2). Care was taken in order to use ramets of similar size and bearing similar numbers of active male zooids. The colonies were placed vertically in a 300 ml glass vessels and exposed to light for 1 hour. The colonies were then removed from the vessels and water samples of 30 ml were taken and sperm counted (see Appendix 2). Sperm release was expressed as the number of sperm per ml per active male zooid.

4.2.2 Fertilization success

Allosperm suspensions were obtained using the dark/light reaction (see Appendix 2). Receptor colonies were then exposed to the allosperm suspension for 1-2 hours, depending on the experiment. As the presence of food induces lophophore protrusion (personal observation), R. reticulata cells were added to the experimental arenas at a final concentration of 10-20 cells·µl^{-1}. Sperm concentration was assessed before experimentation in order to ensure levels were not limiting for fertilization (see Appendix 2). Moreover, in preliminary trials sexual compatibility between the receptor and donor clones was verified. Larvae were not produced by the clones used in the experiments over the previous years were the colonies were kept in reproductive isolation. Number of oocytes produced in each receptor colony was recorded and used to assess the effect of the experimental treatments on fertilization success and hence allosperm capture. Fertilization success was expressed as the number of oocytes per active autozooid at the moment that the receptor colonies were exposed to allosperm suspensions. Active autozooids were considered to be those zooids with a fully developed polypide potentially able to protrude the lophophore and to generate feeding currents. Receptor colonies were exposed to similar concentrations of sexually competent allosperm suspensions for 2h. Once the receptor colonies had been exposed to the allosperm suspensions, they were carefully cleaned with FSW for 10 minutes and then allocated individually to 300 ml flat bottomed glass vessels under standard culture conditions (see Appendix 1). The number of oocytes was noted daily until no more appeared. At the first time each oocyte was observed inside the female coelom, the feeding activity of the autozooids beneath the female zooid was scored. The physiological state of the nearest 4 autozooids placed below each female bearing the oocyte were considered. Those oocytes above at least one active autozooid were cored as "+", those above 4 autozooids with a regressing or regenerating polypide were scored as "-". To assess the position of the oocytes in the colonies camera lucida drawings were made daily. One ramet of each experimental clone was kept in reproductive isolation, to serve as control for self-fertilization.

4.2.2.1 Experiment 1: the effect of number of feeding zooids on fertilization success

The effect of feeding activity on fertilization was examined by exposing to allosperm colonies of similar size but differing in the proportion of active autozooids. In order to produce colonies similar in size but with different amount of active autozooids, two approaches were used. In the first, colonies were deprived of food for four days and maintained in FSW, so forcing the active autozooids to degenerate. After this period of time the colonies were returned to the standard culture conditions. Under these conditions most of the zooids in the colonies degenerated and became unable to protrude their lophophores. In the second approach, half of the active autozooids in the colonies were carefully destroyed using a needle. Ramets of four different clones were used as receptor colonies and ramets of sexually competent clone were used as allosperm donors. Once the allosperm suspensions were obtained the receptor colonies were assigned randomly to the following treatments: receptor colonies bearing most of their zooids as active autozooids (Treatment 1); receptor colonies forced to degenerate (Treatment 2); receptor colonies with half of the active autozooids destroyed (Treatment 3) and receptor colonies with all the autozooids destroyed (Treatment 4).

4.2.2.2 Experiment 2: the effect of lophophore protrusion on fertilization success

The effect of lophophore activity on fertilization was examined by exposing colonies of similar size but differing in the proportion of active autozooids able to protrude their lophophore. In order to prevent lophophore protrusion, a small piece of soft 2 mm thick polyethylene sheet was clipped on top of part of the receptor colonies. Virgin ramets of the same clones involved in the previous experiments were used and the same clone used as allosperm donor. Four ramets from each clone were used and assigned randomly to the following treatments: receptor colonies without obstruction for lophophore protrusion (Treatment 1); receptor colonies with obstruction for lophophore protrusion in half of the colony (Treatment 2) and receptor colonies with total obstruction for lophophore protrusion (Treatment 3).

4.2.2.3 Experiment 3: the effect of water flow on autozooid lophophore protrusion

The effect of water flow on lophophore protrusion was studied using the same small-scale flume described in the sperm release experiments. A stereo microscope (Wild M3) was mounted above the flume and observations of protruded lophophores from active autozooids were counted in a section of 25 mm^2 (ca. 180 basal zooids). Observations were carried out using four clones, one ramet from each being assigned to each experimental water flow condition. In the present experiment, the same water flows conditions described in the sperm release experiments were studied. Sections of acetate sheet bearing the ramets were placed in the bottom of the flume at 10 cm from the water inlet using double-sided adhesive tape to keep the sheet in position. Care was taken to use colonies similar in size and bearing mainly active autozooids. The colonies were maintained in the flume for 10 minutes for acclimation and then three observations of the number of protruded lophophores were assessed at intervals of 10 minutes. Number of protruded lophophores was expressed as a proportion of the total number of active zooids inside the sampling area.

4.2.2.4 Experiment 4: the effect of water flow on fertilization success

The effect of water flow on fertilization success was studied by exposing receptor colonies to allosperm suspensions at different flow velocities. Sections of acetate sheet bearing the receptor colonies were placed in the bottom of the flume at 10 cm from the water inlet using double sided adhesive tape to keep the sheet in position. A long acetate strip attached to the acetate bearing the experimental colony was used to remove the colony from the flume as soon the experimental treatment had finished. Care was taken to use colonies similar in size and bearing mainly active zooids. The allosperm suspensions were then poured from a beaker into the flume and the required water velocity applied. Four different clones were used in the present study, one ramet of each clone being assigned randomly to each water flow condition: still water, low, medium and high flow condition. The receptor colonies were maintained in the flume for 1 h in contact with the compatible allosperm suspension, then removed and cleaned for 10 minutes with FSW before being assigned to the culture vessels.

4.3 Results

4.3.1 Sperm release

Sperm were released by most of the males within the area under observation. Sperm release was always confined to the first 5-10 minutes after the colonies were removed from darkness. Sperm release by individual zooids was observed to last 5 to 15 seconds. Under still conditions, sperm release was accompanied by flicking of the protruded lophophore. When sperm release occurred in moving water, sperm were released from the tip of the protruded lophophore, which was bent in the direction of water movement. Under these conditions, sperm were seen leaving the protruded male lophophores like a smoke plume downwind of a chimney (Plate 1)

4.3.1.1 Experiment 1: the effect of water flow on sperm release

Sperm release was significantly and negatively correlated with water flow (Table 1). More sperm per ml per active male were found in still water and low water flow conditions (Fig. 1). Almost twice as much sperm were released in still water and in the low water flow condition than in medium flow. Drastic diminution of sperm release occurred in high water flow (Fig. 1). There was a significant genotype-water flow interaction, although genotype itself had no significant effect on sperm release (Table 1).

4.3.1.2 Experiment 2: the effect of water flow on male lophophore protrusion

Male lophophore protrusion was more frequent in still water and in low water flow (Table 2). However, in medium flow male lophophore protrusion was reduced by a factor of 6, and no protrusion was observed in high water flows (Fig. 2). There was a significant genotype-water flow interaction, but no significant effect of clone on male lophophore protrusion was found (Table 2).

4.3.1.3 Experiment 3: the effect of lophophore protrusion on sperm release

Sperm release was significantly related to lophophore protrusion (Table 3). Sperm release decreased progressively with increasing prevention of lophophore protrusion (Fig. 3).

4.3.2 Fertilization success

Oocytes were found in the receptor colonies in all the experimental colonies exposed to allosperm suspensions, but not in the control colonies.

4.3.2.1 Experiment 1: the effect of number of feeding zooids on fertilization success

The number of oocytes per colony was significantly affected by the number of active autozooids present in the receptor colonies. Although there was no significant effect of clone, there was a significant clone-active autozooids interaction (Table 4). Highest numbers of oocytes were found in healthy receptor colonies bearing mainly active autozooids, these values being nearly twice these found in half healthy receptor colonies (Fig. 4). When colonies bearing recycling zooids were used as receptor colonies, few oocytes were found. No oocytes were found in receptor colonies were the autozooids were destroyed (Fig. 4). In all the treatment where oocytes were found, in general more that 70 % of the oocytes were found in ovicells placed on top of active zooids (Fig. 5). Fertilization success was found not to be significantly affected by colony status, clone, and the interaction of colony status and clone (Table 5), but overall had values of about 0.1 oocytes per active autozooid (Fig. 6).

4.3.2.2 Experiment 2: the effect of lophophore protrusion on fertilization success

The number of oocytes was significantly affected by prevention of lophophore protrusion ($H=9.85$; $DF= 2$; $P<0.01$). Two to five times more oocytes were found in this treatment than in half and totally covered receptor colonies (Fig. 7). The lowest values of oocytes were found in colonies in which lophophore protrusion was totally prevented (Fig. 7). In the three experimental conditions, more than 75% of the oocytes were found in the receptor colonies inside ovicells on top of active zooids (Fig. 8). Values of fertilization success were significantly affected by prevention of protrusion ($H=7.50$; $DF=2$; $P<0.05$). Maximum values of fertilization success were found in the treatments where prevention of protrusion was absent or confined to half of the receptor colony (Fig. 9). When protrusion was totally prevented the minimum values of fertilization were obtained (Fig. 9).

4.3.2.3 Experiment 3: the effect of water flow on autozooid lophophore protrusion

The proportion of protruded lophophores was significantly affected by water flow ($H=33.39$; $DF=3$; $P<0.0001$). The maximum proportions of protruded lophophores were observed at still, low and medium water flow conditions (Fig.10). When high water flow conditions were used the lowest proportions of protruded lophophores were found.

4.3.2.4 Experiment 4: the effect of water flow on fertilization success

The number of oocytes was significantly affected by the water flow at the time of fertilization ($H=8.68$; $DF=3$; $P<0.05$). Similar numbers of oocytes were produced when fertilization occurred in still water and in low and medium flows (Fig. 11). When colonies were exposed to sperm suspensions at high water flow, the lowest numbers of oocytes were found (Fig. 11). In all the experimental conditions, close to 80% of the oocytes were positively associated with number of active autozooids (Fig. 12). Fertilization success was significantly affected by water flow ($H=7.95$; $DF=3$; $P<0.05$), with consistently high values found in still water and in low and medium flow (Fig. 13). When receptor colonies were exposed to allosperm suspension at high water flow, the lowest values of fertilization success were found (Fig. 13).

4.4 Discussion

Release of sperm through pores at the tips of lophophore tentacles has been documented for several bryozoans (Silén, 1972). The present study is the first report that this phenomenon also occurs in C. hyalina. Male lophophore protrusion occurred only in slowly moving or stagnant conditions after the darkness-light treatment, perhaps explaining why male protrusion has not often been observed (Cancino, 1983; DJ Hughes 1987). Moreover, because those studies were designed to investigate problems at the colonial and population levels, observations at the zooid level were probably missed. Finally, problems associated with the magnification and optic of the instruments used in the observations cannot be discard.

High sperm counts in conditions favourable for lophophore protrusion plus direct observation of sperm clouds discharged from the protruded lophophore confirm that sperm release does indeed occur through this organ. Protrusion of groups of male lophophores suggests that sperm release in C. hyalina could be synchronized within colonies. Synchronous release of gametes by an individual could influence fertilization success (Sewell and Levitan, 1992). As relatively high concentrations of sperm may be of key importance in sperm competition (Levitan, 1995), synchrony could enable more concentrated sperm suspensions to be released close to receptive females. Although most evidence of synchronized spawning came from groups with external fertilization (see Levitan, 1995 for a review), intense sperm competition and also sperm dilution could be invoked to explain intra-colonial synchrony of sperm release in C. hyalina. Field manipulations of C. hyalina have shown that distant colonies have reduced fertilization success when near colonies are not presents (Yund and McCartney, 1994). Hence, it may be inferred that donor colonies (i.e. colonies acting as sperm source) close to receptor colonies (i.e. colonies acting as sperm receivers) have the opportunity to deliver more concentrated sperm suspensions to receptor colonies than is possible for distant colonies. Colonies of C. hyalina are present at high densities on kelps and fucoids and frequently grow into contact with each other (see Chapter 8). Therefore, competition between colonies acting as sperm donors is possible. Sperm release under still water, together with intra-colonial synchrony, could be interpreted as a mechanism in C. hyalina to avoid sperm dilution and maximize fertilization. Sperm release under low and medium flows would

promote the dispersal of sperm. This could be advantageous by reducing the chance of inbreeding and associated inbreeding depression. In several species of bryozoans there is a general tendency for larvae to settle gregariously, so minimizing inter-specific competition (Stebbings, 1973; Seed and O'Connor, 1981; Ryland, 1976), escape predation (Keough, 1984) and facilitate cross-fertilization among simultaneous hermaphrodite colonies (Hayward and Ryland, 1975; Keough, 1984). Settlement of genetically related colonies of C. hyalina has not yet been demonstrated in natural populations. However, recently it has been recognized that genetic differences in natural population of the Welsh C. hyalina are present even at relatively small spatial scales (Goldson, 1998). In Bugula neritina, sibling larvae settled in an aggregated pattern while unrelated larvae settled randomly (Keough, 1984). However, similar studies with C. hyalina have shown that full and half sib larvae do not tend to settle gregariously (unpublished own results). Therefore, it is to be expected that colonies growing on their natural substrata are randomly arranged with respect to genetic relatedness. The aggregation of unrelated colonies would facilitate cross-fertilization, so minimizing potential inbreeding depression.

All experiments in the present study used virgin colonies and sexually compatible allosperm suspensions. Fertilization success therefore should have been a response to the experimental treatment. In Experiments 1 and 2 the high values of fertilization success associated with treatments affording maximum opportunity for lophophore protrusion can probably be explained by an active role of the protruded lophophore of autozooids in sperm capture or concentration.

In C. hyalina retention efficiency or the proportion of seston particles captured at the filtration surface in relation to the total of those which approach it (Wildish and Kristmanson, 1997) decays rapidly when particle diameter is below 6 µm (Riisgård and Manríquez, 1997). The head of mature sperm in C. hyalina is 10 µm long with a maximum diameter of 0.5 µm and the tail is 40-50µm long (Manríquez and Bishop personal observations). Although the diameter of the head is below the critical retention size for the filtering mechanism of C. hyalina, pumping in C. hyalina is able to entrain particles placed some micrometers above the protruded lophophore (see Fig 6 C and D in Riisgård and Manríquez, 1997). Therefore it is possible that the lophophore current could play a role in bringing allosperm close to receptor colonies. Moreover, considering the

total length of sperm in C. hyalina (ca. 50-60µm), it is possible that the direct contact between swimming sperm and protruded lophophores could facilitate their capture. Sperm adhesion to lophophore tentacles has been described by Marcus (1926) for Farrella repens, Silén (1966) for Electra posidoniae, and later by Temkin (1996) for Membranipora membranacea. Thus, it is possible that the process described by Silén represents the predominant situation in bryozoans with internal fertilization. In the present study the association between oocytes and underlying active autozooids supports this suggestion.

The presence of oocytes associated with female zooids in areas in which lophophore protrusion was prevented can be explained by sperm reaching this area. Sperm could reach the area through the small gap between the colony and the polyethylene sheet. Moreover in preliminary trials using a cover slip to prevent lophophore protrusion, ciliary activity associated with the tips of the barely protruded lophophores was able to entrain R. reticulata cells present in the vicinity. All these results are in agreement with the high association of oocytes with the proximity of active autozooids in all the experimental receptor colonies. Since intra-colonial sperm movement is restricted to short distances within the colonies (see Chapter 3), presence of embryos in areas distant from the barely protruded lophophores cannot be attributed to sperm transfer from this area to areas with prevention of lophophore protrusion.

Cancino (1983) noted restriction of reproductive zooids to areas with mainly active zooids. This suggest that sperm entrance is related to active autozooids, perhaps entering the coelom of an autozooid via the dorsal coelomopore as has been suggested by Ostrovsky (1998) and demonstrated by Temkin (1996). Marcus (1938) proposed sperm entrance through expanded lophophores and migration to female zooids via the funicular system. However, DJ Hughes (1987) raised doubts over this idea because of the existence of a cellular plug associated with the pore plates (Banta, 1969; Gordon, 1975). The observed flicking and bending of protruded male lophophores within the funnel of the nearest protruded autozooid lophophore in still water conditions could assist sperm broadcast, since the sperm could be expelled with uncaptured particles. In C. hyalina uncaptured particles usually leave the lophophore with the excurrent current at velocities of approximately 1.5 $mm \cdot s^{-1}$ (see Riisgård and Manríquez, 1997), thus under low water movement in the field this mechanism could favour sperm dispersion from the paternal

colony. The negative effect of increasing water velocity on the incidence of lophophore protrusion has been also described in Chilean C. hyalina (Muñoz, 1996). Highest values of protrusion were found within the range of velocities likely to be experienced in the field (Muñoz, 1996). To date, no information is available on water velocities experienced by colonies of C. hyalina growing on their natural substrata in the Menai Strait (i.e. kelps and fucoids). Organisms associated with seaweeds growing in nearshore environments however are subject to both transient high water velocities and desiccation. Growth of benthic filter feeders generally is a function of current speed (Sanford et al., 1994), possibly because tidal currents supply the phytoplanktonic food (Mann and Lazier, 1996). In encrusting bryozoans, however increased water flow can be detrimental to feeding success (Okamura 1984, 1985, 1988, 1992), perhaps by disturbing the protruded lophophores (Muñoz, 1996). However in low water velocities the development of a thick diffusion boundary layer reduces transport of food or nutrients. This has been described for algae where low water velocities (ca. <4-6 $c \cdot ms^{-1}$) reduce the transport of essential inorganic nutrient to the plant surface with consequences on seaweed productivity (Wheeler, 1980). On high spring tides, tidal current in Menai Strait can reach maximum values between 0.8-1.2 $m \cdot s^{-1}$ (Harvey, 1968; Owrid and Ryland, 1993), however low water velocities are expected during slack water (Sherwin, 1998). Recent studies have shown that bryozoan feeding rates increase with increasing flume flow (Larsen et al., 1998). The findings were interpreted as a result of thinning of the boundary layer and can increase in flux of particles around the colonies. However, the water velocities used by Larsen et al. (1998) were less than those used in the present study and did not impede lophophore protrusion.

In conclusion, the present study has shown that sperm are released by the male lophophores, and that under laboratory conditions sperm are released mainly under still and low water flow. Under these conditions C. hyalina could increase fertilization success by avoiding dilution of sperm due to turbulence (Denny, 1988). Moreover, sperm could remain trapped within the relatively thick boundary layer associated with low water velocities. The fertile life of C. hyalina sperm is about 1 h in still water (see Chapter 2). Thus it is plausible that sperm moving within a thick boundary layer may avoid wastage associated with transport outside this zone, without compromising their viability. Water flow conditions favouring sperm release also enhance fertilization success, possibly

through the effect on feeding activity. These results suggest that factors that can cause sperm dilution (e.g. water flow) could be as much as severe in brooders that concentrate sperm from the water as in free-spawner marine organisms (Yund, 2000).

If sperm enter receptor zooids through the supraneural pore in the base of the tentacles (Marcus, 1938), the inhalant current itself could assist the collection of allosperm. Such a mechanism could explain the observed distribution of the oocytes in ovicells on top of active feeding zooids. These findings do no preclude the possibility that entry of sperm takes place directly through the female zooids as suggested by Ostrovosky (1998). However, occurrence of oocytes in ovicells that were not yet developed when allosperm were administrated (see Chapter 3) suggests that sperm entry takes place through active feeding zooids.

Recent studies have shown a tendency to miniaturization in colonies of Membranipora membranacea exposed to high water flows (Okamura and Partridge, 1999), suggesting that this reduction in colonial cover could have negative effects on fertilization success. It therefore seems that the influence of water flow on fertilization success is an important selective pressure acting on life-history traits in sessile organisms with broadcast sperm and internal fertilization. It will be important to assess the significance of these results in natural circumstances, in order to further understand the reproductive biology of marine invertebrates with internal fertilization via broadcast sperm.

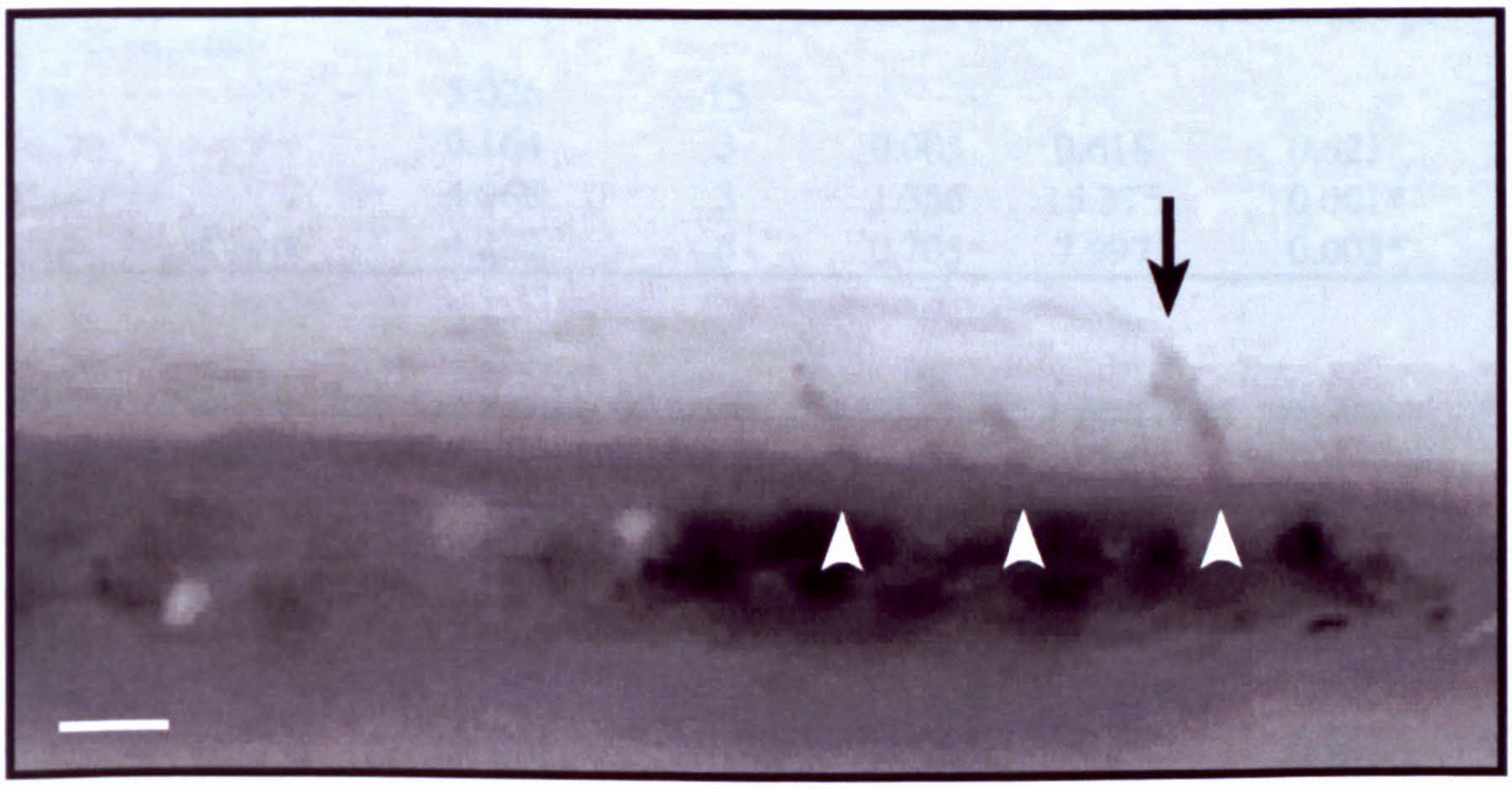

Plate 1: Cloud of sperm being released from the protruded male tentacles in a colony of C. hyalina. White arrows indicate the base of the male tentacles and the black arrow the cloud of sperm. Negative Image. Scale bar = 150 μm.

Table 1: ANOVA table for results of sperm release under 4 levels of water flow (see Fig. 2). Data are square root transformed.

Source	SS	DF	MS	F	p
Sperm release					
Total	16.27	19			
Clone	2.46	4	0.62	1.34	0.33
Treatment	8.29	3	2.76	6.00	0.01*
Clone x Treatment	10.75	7	1.54	3.34	0.03*

Table 2: ANOVA table for results of male lophophore protrusion under 4 levels of water flow (see Fig. 3). Data are square root transformed.

Source	SS	DF	MS	F	p
Male protrusion					
Total	5.026	15			
Clone	0.164	3	0.005	0.618	0.621
Treatment	4.068	3	1.356	15.375	0.001*
Clone x Treatment	4.232	6	0.705	7.997	0.003*

Table 3: ANOVA table for results of sperm release under 3 levels of prevention of lophophore protrusion (see Fig. 4). Data are square root transformed.

Source	SS	DF	MS	F	p
Sperm release					
Total	7.569	11			
Clone	0.148	3	0.049	0.324	0.809
Treatment	6.506	2	3.253	21.333	0.002*
Clone x Treatment	6.654	5	1.331	8.727	0.010*

Table 4: ANOVA table for results of total number of oocytes for colonies with different physiological status (see Fig. 5). Treatment 1 healthy colonies, Treatment 2 recycling colonies, Treatment 3 half healthy colonies and Treatment 4 dead colonies. Data are square root transformed.

Source	SS	DF	MS	F	p
Number of oocytes					
Total	490.95	15			
Clone	22.40	3	7.47	0.582	0.642
Treatment	345.68	3	115.23	8.981	0.005*
Clone x Treatment	375.48	6	62.58	4.878	0.017*

Table 5: ANOVA table for results of fertilization success (FS = number of oocytes per active autozooid) for colonies with different physiological status (see Fig. 6). Treatment 1 healthy colonies, Treatment 2 recycling colonies, Treatment 3 half healthy colonies and Treatment 4 dead colonies. Data are square root transformed.

Source	SS	DF	MS	F	p
FS (square root transformed)					
Total	0.356	11			
Clone	0.086	3	0.029	0.76	0.557
Treatment	0.043	2	0.021	0.57	0.593
Clone x Treatment	0.129	5	0.258	0.68	0.654

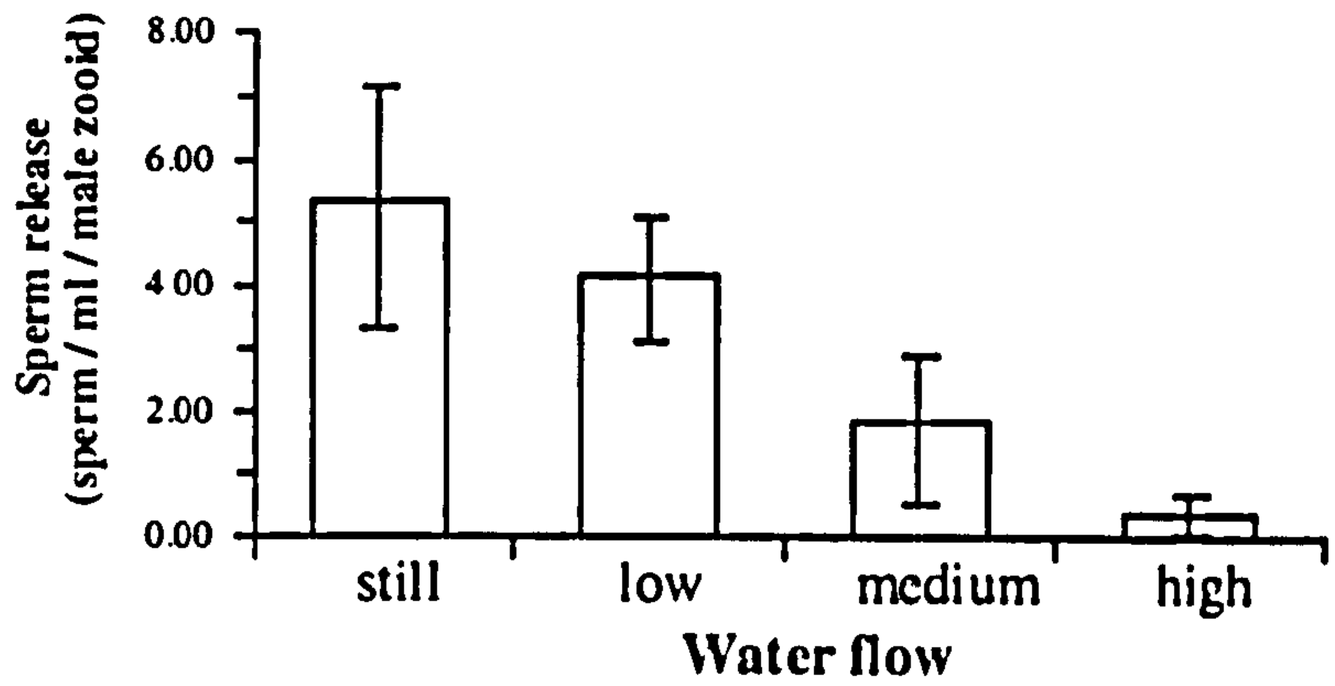

Figure 1: Mean sperm release, Experiment 1. The effect of flow on sperm release in C. hyalina. Number of sperm per ml and per male zooid under levels of water flow. Bars are one SE.

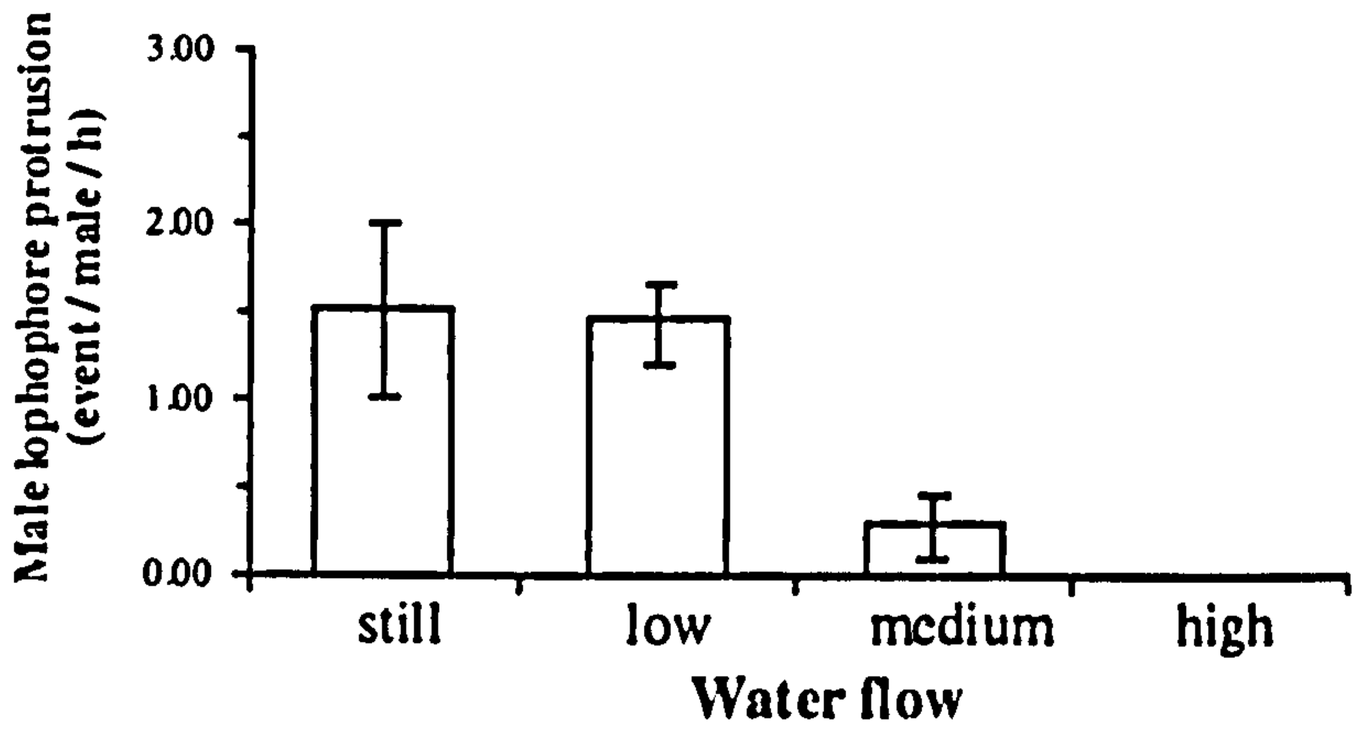

Figure 2: Mean sperm release, Experiment 2. The effect of flow on male lophophore protrusion in C. hyalina. Protrusions per male and per hour under four levels of water flow. Bars are one SE.

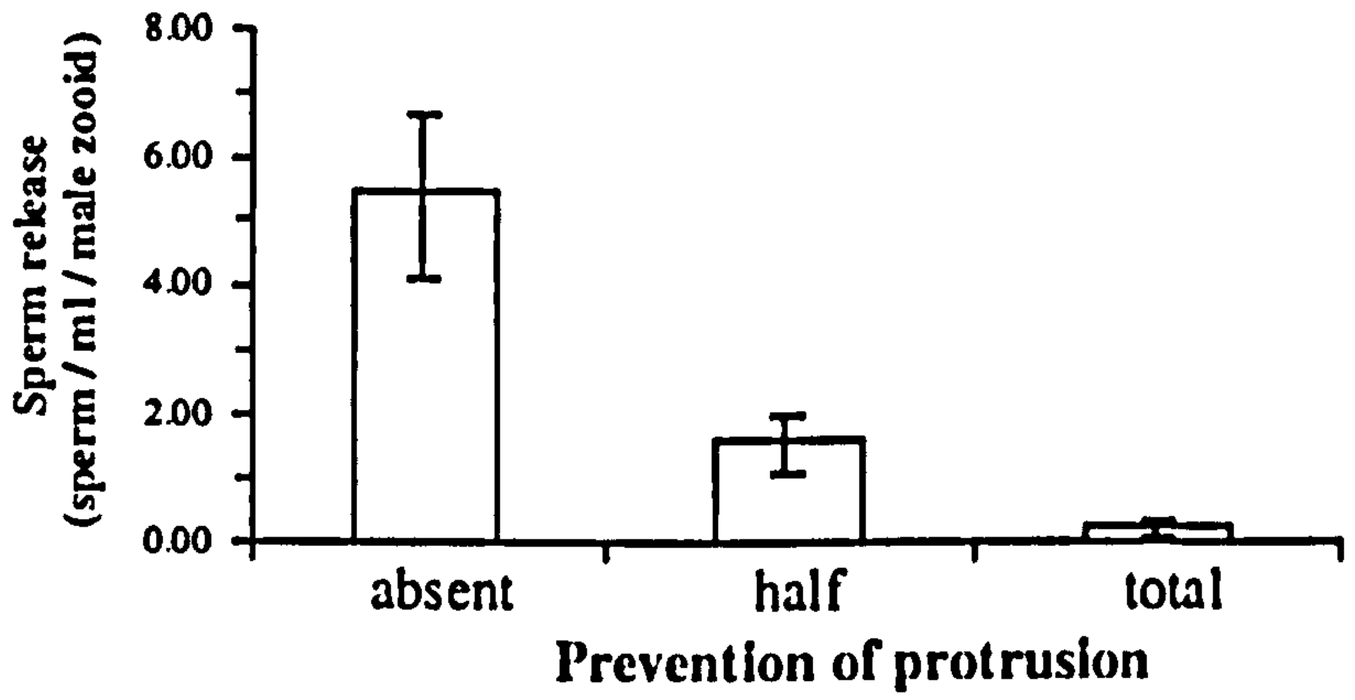

Figure 3: Mean sperm release, Experiment 3. The effect of lophophore protrusion on sperm release in C. hyalina. Number of sperm per ml under different experimental levels of lophophore protrusion. Bars are one SE.

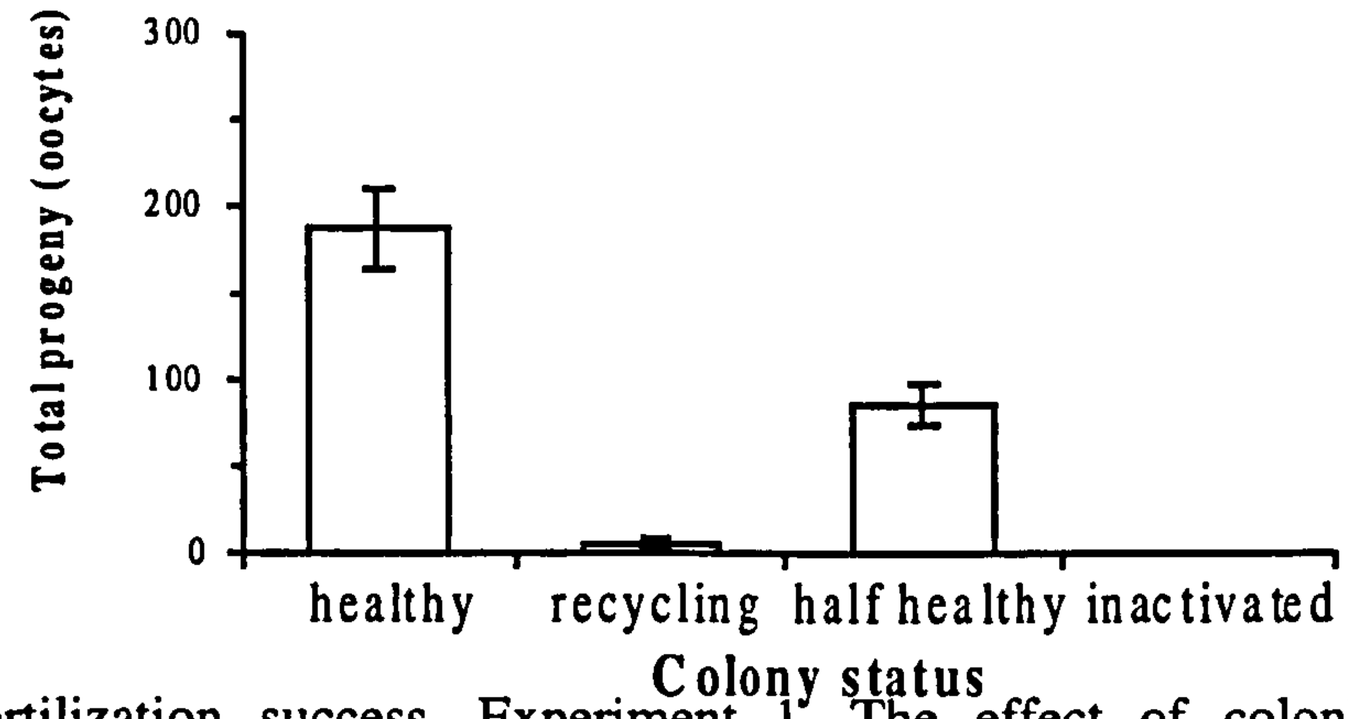

Figure 4: Fertilization success, Experiment 1. The effect of colonial status on production of progeny in C. hyalina. Number (mean ± SE) of oocytes sired by colonies under four experimental levels of autozooid activity.

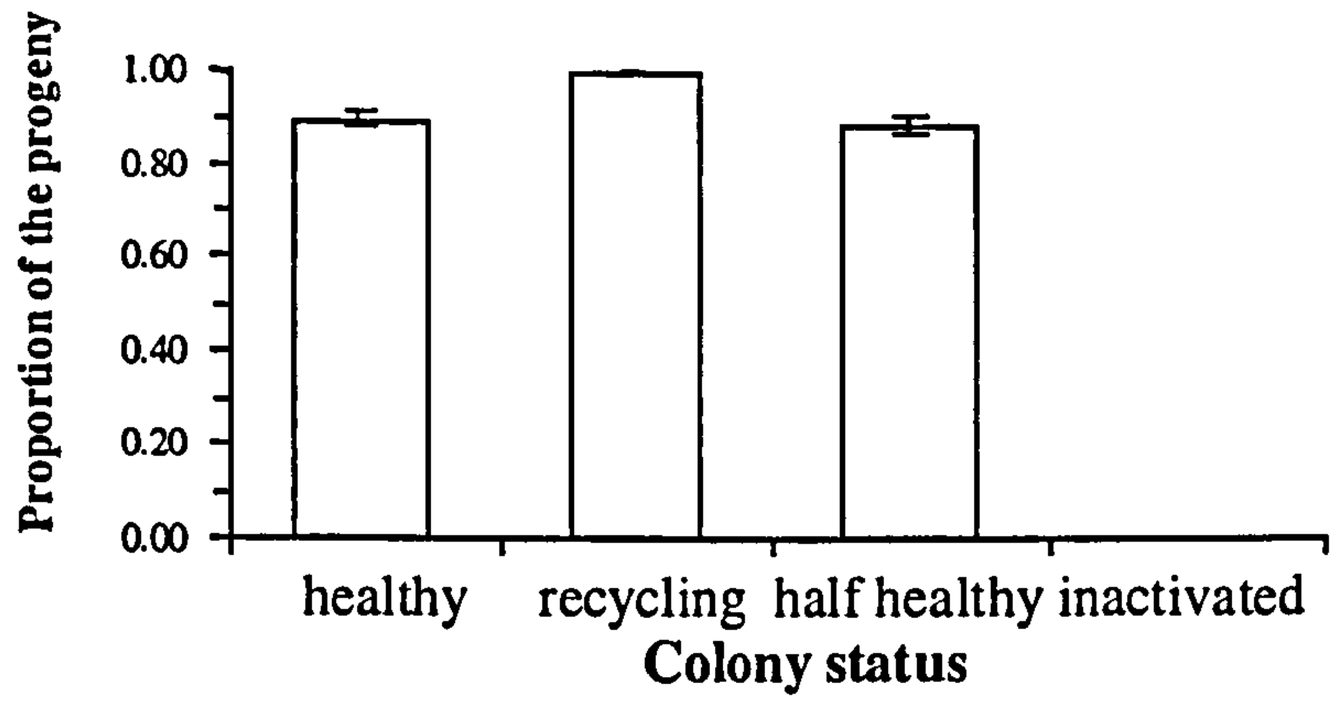

Figure 5: Fertilization success, Experiment 1. The effect of colonial status on the spatial distribution of oocytes in colonies of C. hyalina. Proportion (mean ± SE) of oocytes placed in ovicells above active autozooids under four experimental levels of autozooid activity.

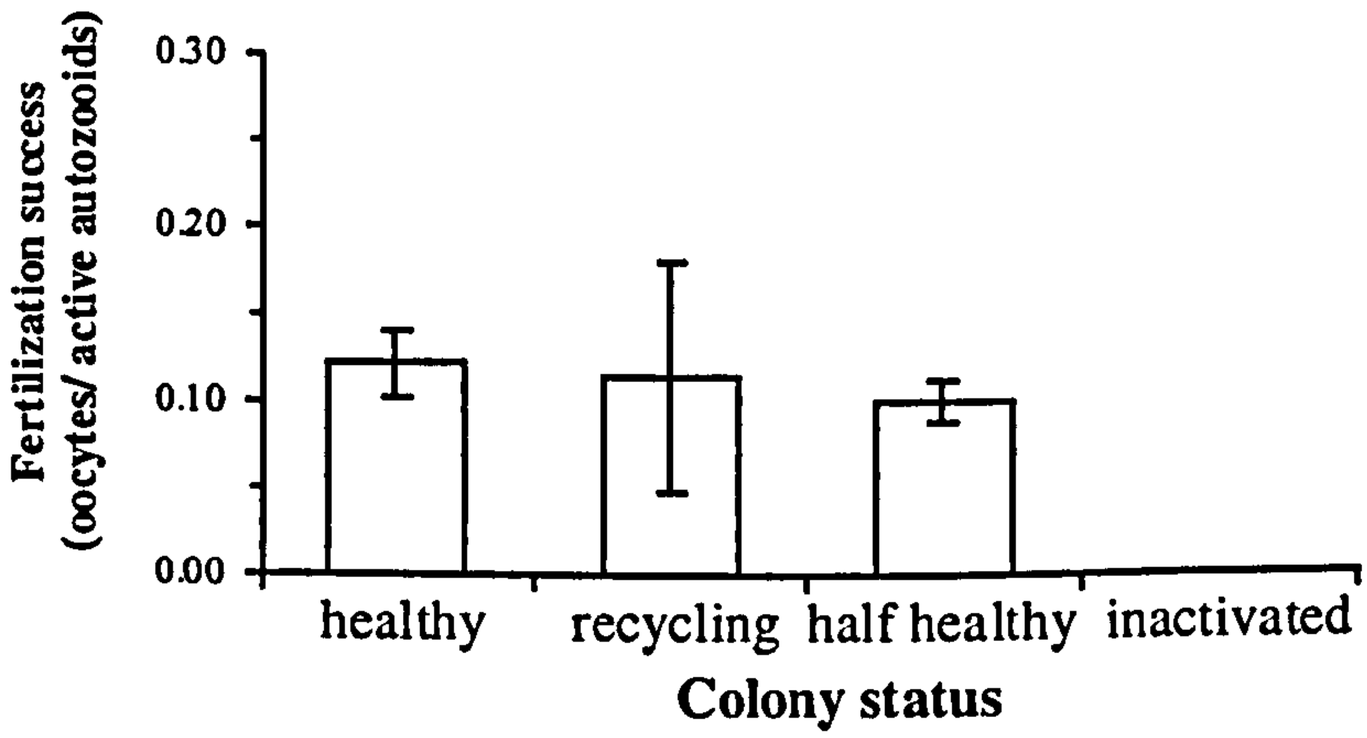

Figure 6: Fertilization success (FS), Experiment 1.The effect of colonial status on fertilization success in colonies of C. hyalina. FS (mean ± SE) in colonies of C. hyalina under four experimental levels of autozooid activity.

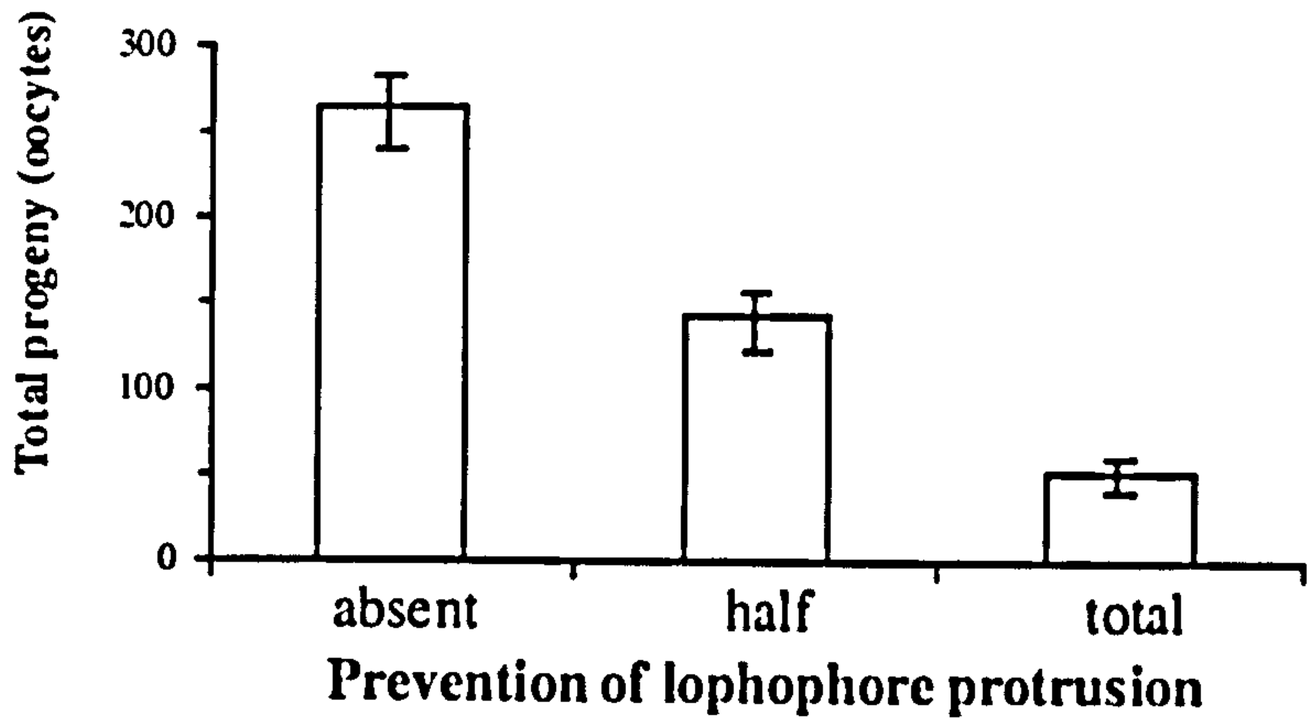

Figure 7: Fertilization success, Experiment 2. The effect of prevention of lophophore protrusion on production of progeny in C. hyalina. Number (mean ± SE) of oocytes produced by colonies under three experimental levels of lophophore protrusion.

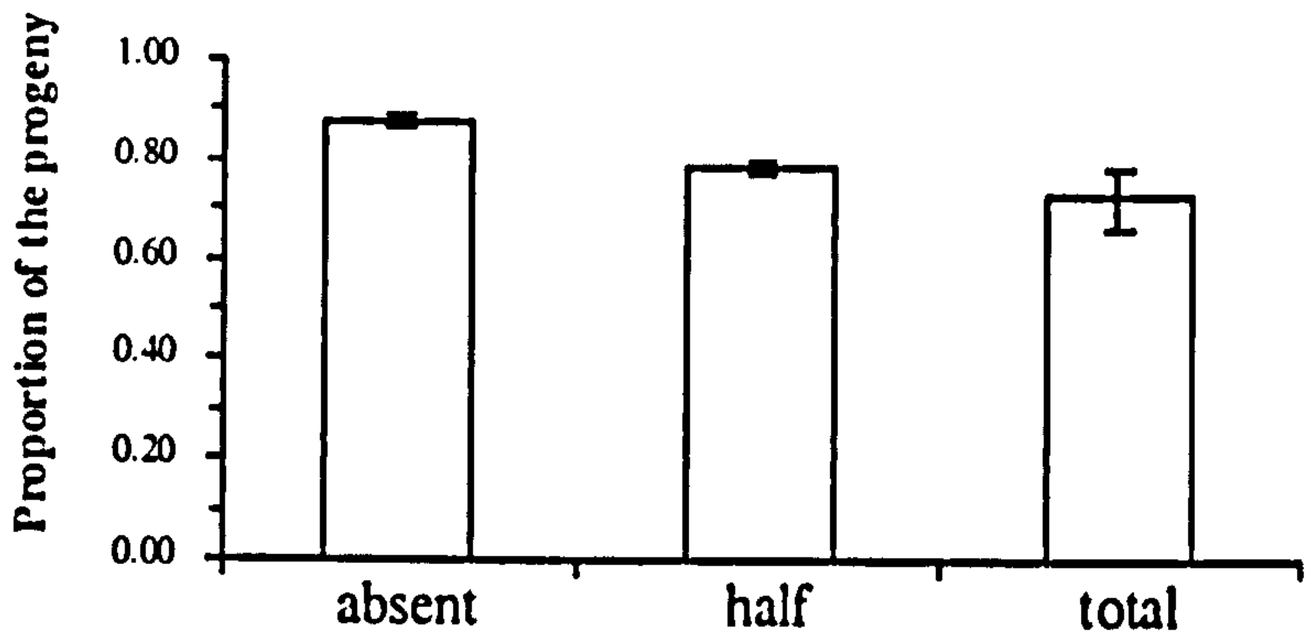

Figure 8: Fertilization success, Experiment 2. The effect of prevention of lophophore protrusion on the spatial distribution of the oocytes in colonies of C. hyalina. Proportion of oocytes (mean ± SE) placed in ovicells above active autozooids in colonies under three experimental levels of lophophore protrusion.

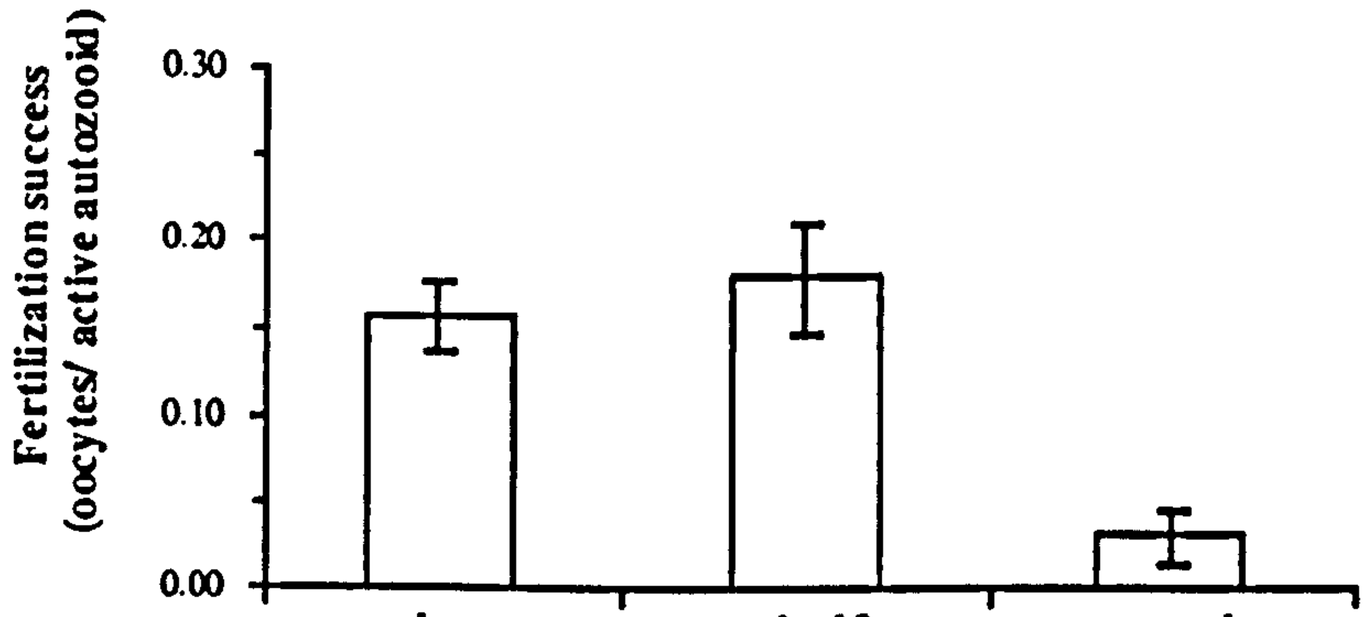

Figure 9: Fertilization success, Experiment 2. The effect of prevention of lophophore protrusion on fertilization success (oocytes per active autozooid) in colonies of C. hyalina. FS (mean ± SE) in colonies of C. hyalina under three experimental levels of lophophore protrusion.

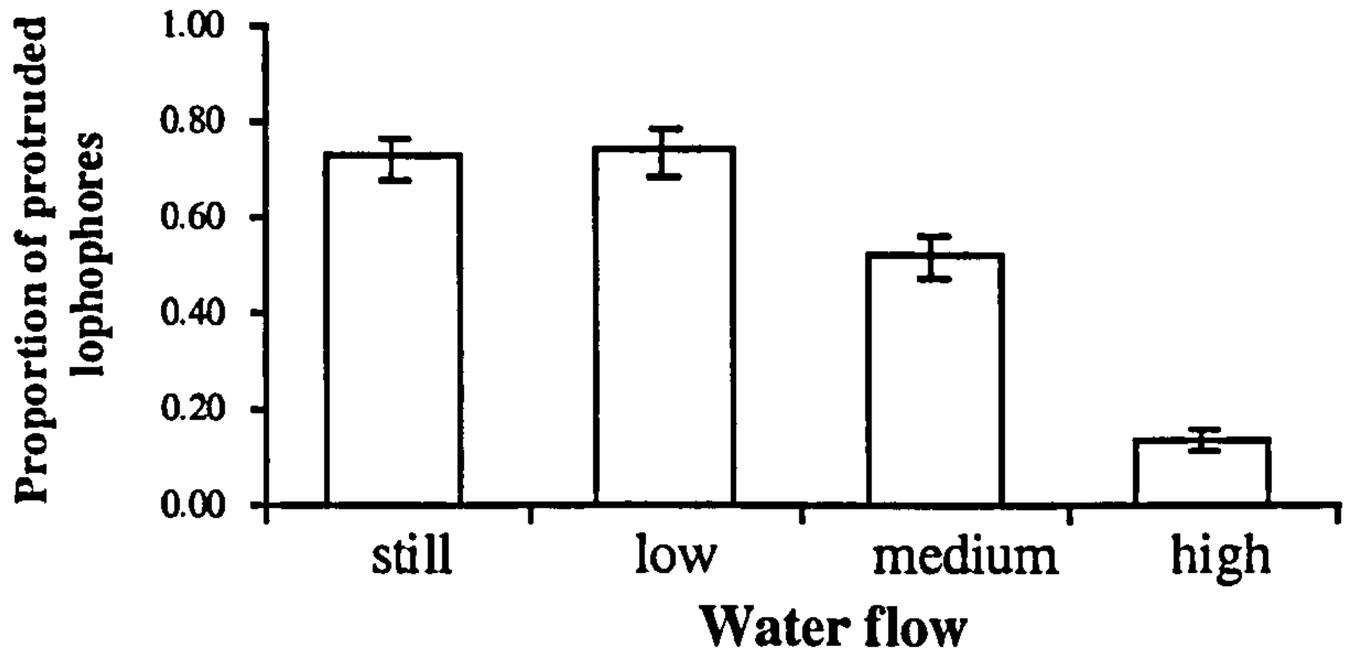

Figure 10: Fertilization success, Experiment 3. Proportion (mean ± SE) of protruded autozooid lophophores in colonies of C. hyalina under four water flow.

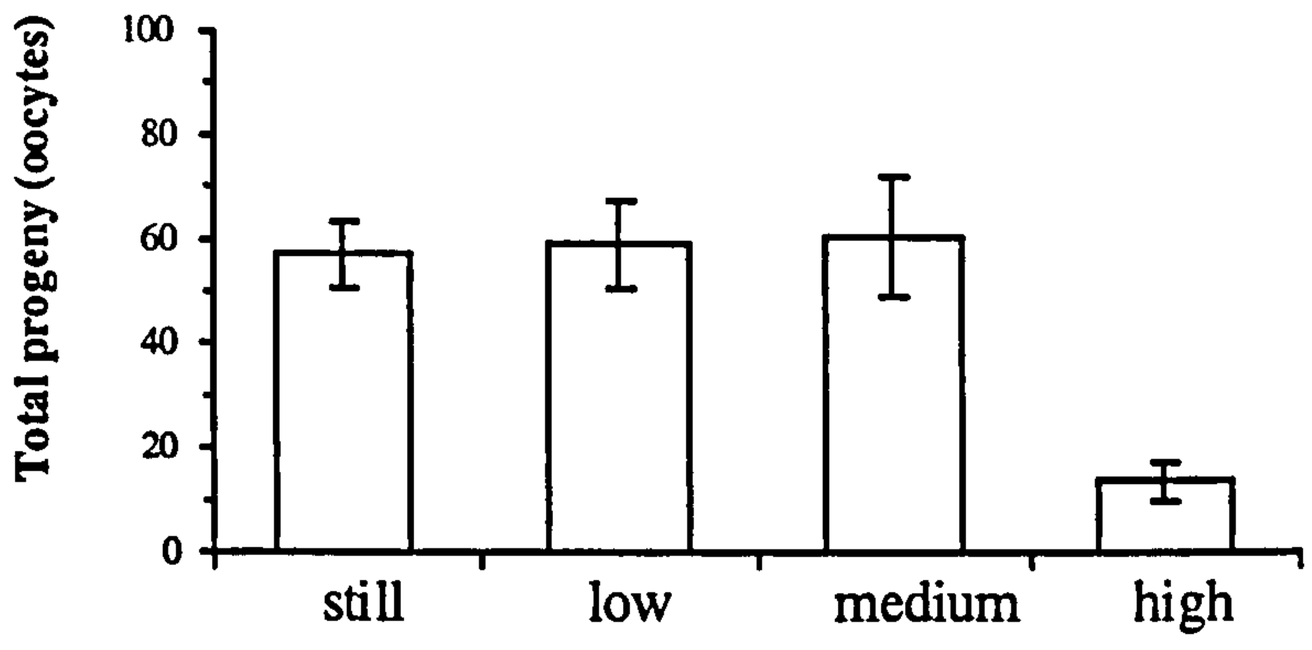

Figure 11: Fertilization success, Experiment 3. The effect of water movement on production of progeny in C. hyalina. Number (mean ± SE) of oocytes sired by colonies under four levels of water flow.

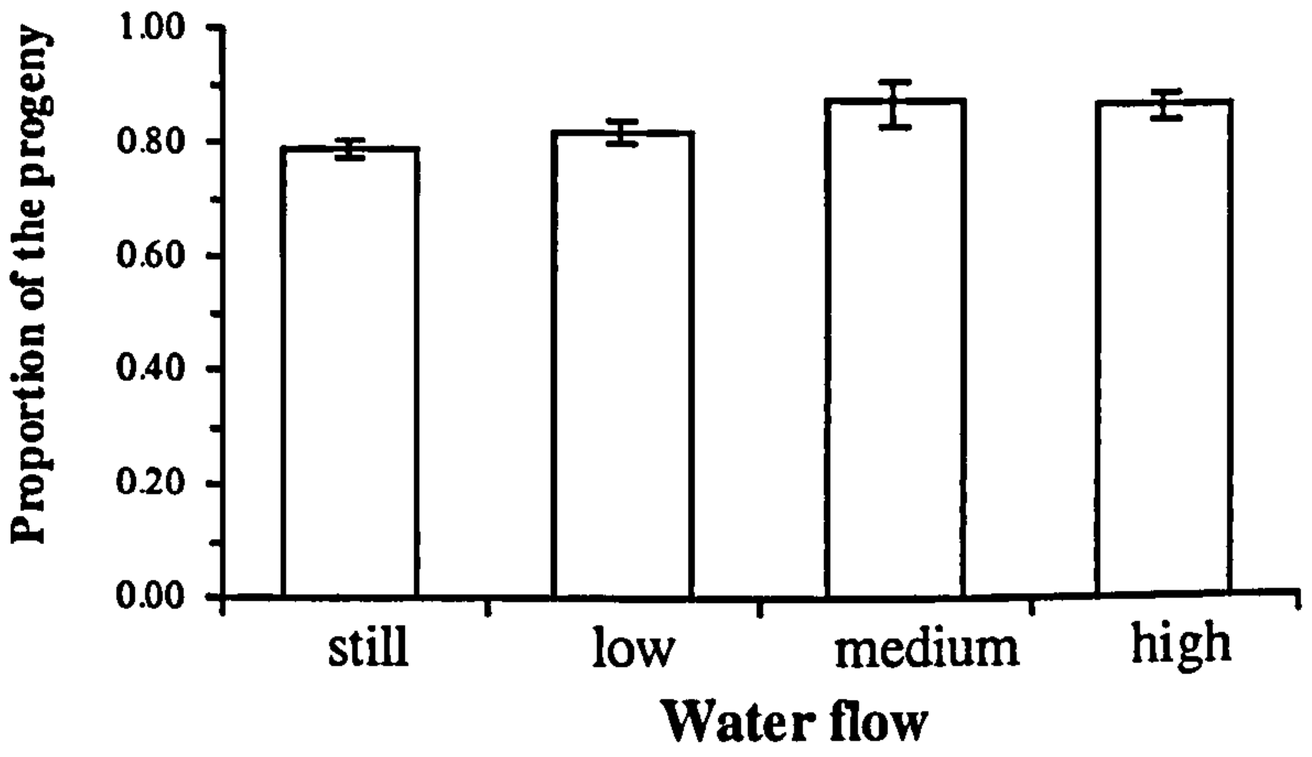

Figure 12: Fertilization success, Experiment 3. The effect of water movement on the spatial distribution of oocytes in colonies of C. hyalina. Proportion of oocytes (mean ± SE) placed in ovicells above active autozooids under four experimental levels of water flow.

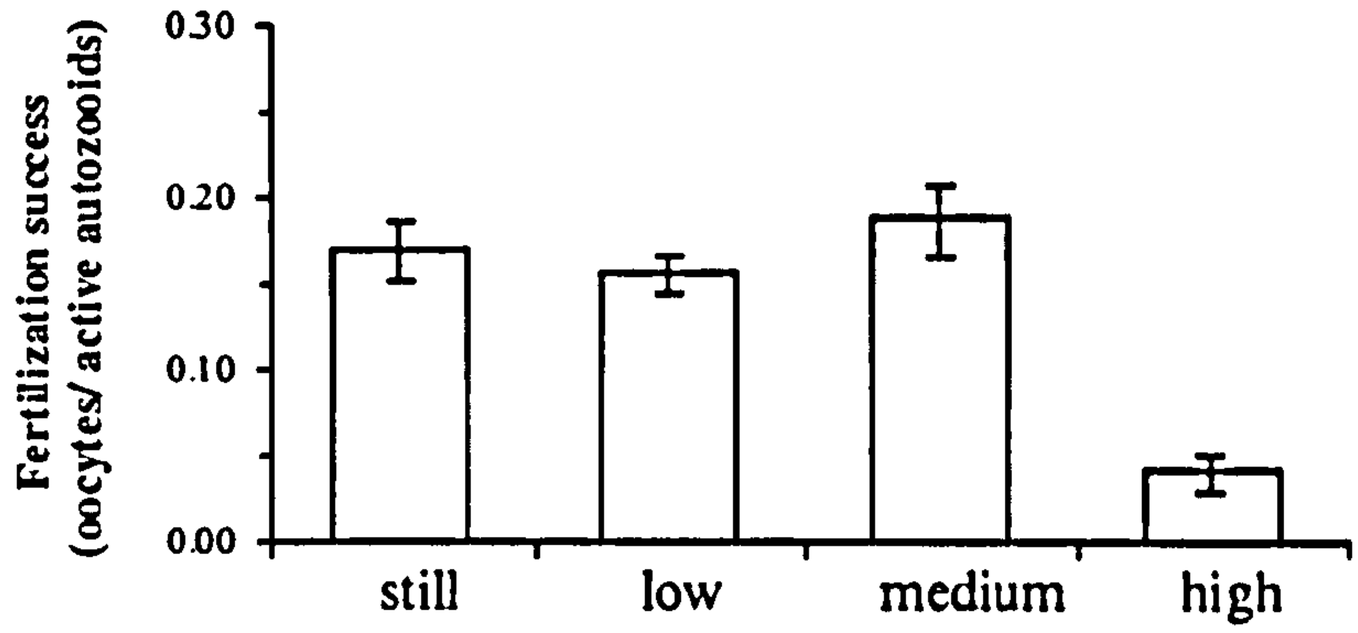

Figure 13: Fertilization success (FS), Experiment 3. The effect of water movement on fertilization success in colonies of C. hyalina. FS (mean ± SE) in colonies of C. hyalina under four experimental levels of water flow.

Chapter 4

Chapter 5:
Sperm competition and female choice

5.1 Introduction

The eggs of marine invertebrates whose sperm are shed into the water are maybe exposed to a mixture of allosperm. Thus, multiple mating is a likely consequence for invertebrates that retain eggs which are internally fertilized by aquasperm. Under this scenario of fertilization of eggs may approximate to a "fair raffle" (Parker, 1990). One male may fertilize a major proportion of the available eggs while other males may father a minority of the offspring. In copulating animals, explanations for multiple copulation include the fertilization hypothesis (Birkhead et al. 1987) and the sperm competition hypothesis (Parker, 1970). The first hypothesis pointed out that in order to ensure fertilization of all their eggs, females copulate several times with a single or multiple partners. The second hypothesis state that in species in which females mate more than once, the females may increase the quality of their offspring by allowing sperm from different males to compete for fertilization.

The sessile bryozoan C. hyalina (L.) is a colonial, hermaphroditic marine filter-feeding organism. Sperm are released into the water through male tentacles (see Appendix 2) and then taken up by receptor colonies. Although the precise route of access for allosperm into the receptor colony is not known, uptake seems to be related to protruded tentacles from the feeding zooids. For cheilostomes, it has been proposed that sperm enter the fertile zooids through the supraneural coelomopore (Silén, 1966), which in C. hyalina is associated with basal autozooids (Marcus, 1938). In their natural habitat, Welsh colonies growing on fronds of Laminaria saccharina and Fucus serratus must be exposed to a mixture of sperm from different potential mates including both selfsperm and allosperm. Competition for fertilization among potential mates therefore could take place. Consequences of sperm competition for fertilization in C. hyalina have been evaluated in the field (Yund and McCartney, 1994). This study found that sperm released by one male colony reduced the number of fertilizations obtained by sperm from another colony.

Sperm competition inside the female reproductive tract can be decided by one of the following three factors: (1) a fair raffle (Parker, 1990), in which the probability of

fertilization is proportional to the number of sperm inseminated by each male; (2) sperm precedence, in which the sperm from either the first or, more commonly, the last male to inseminate the female fertilize the majority of the eggs or; (c) sperm quality, in which the sperm with superior features, such as higher speed, fertilize the majority of the eggs. However, an active role of females exerting control on paternity within their reproductive tract has also been suggested (Birkhead and Møller, 1993; Eberhard, 1996; Bishop, 1996; Bishop et al., 1996). Females are expected to choose mates with characteristics that will enhance their fitness. Males may provide good genes to their offspring, which also should enhance the fitness of the females. Therefore, if females are to make adaptive mate choices, they must assess male traits before or after mating. The value of mate choice depends on the degree of variation in quality among members of the opposite sex (Parker, 1983). If no differences exist and the potential mates are sexually compatible, progeny are expected to be fathered in proportion to sperm abundance.

When internal fertilization occurs, an important prerequisite for many females to select or choose sperm is a mechanism of sperm storage. Moreover, for simultaneous hermaphrodites, if mate choice takes place it is expected to be assortative (Michiels, 1998). Storage of allosperm in C. hyalina was suggested by Cancino (1983) and is demonstrated here (see Chapter 3). First-male sperm priority in C. hyalina has also been demonstrated (see Chapter 6). However, as self-fertilization is a rare event in C. hyalina, the dosage and proportion of allosperm can be manipulated and used to fertilize receptor colonies without the risk of precedence by autosperm (i.e. autosperm displacing allosperm). Moreover, as sperm release in C. hyalina can be triggered by a dark-light shock (see Appendix 1), fresh sperm suspensions can be prepared thereby avoiding effects of sperm ageing (see Chapter 2). Also, dosage can be adjusted to yield similar proportions of sperm, so avoiding competition effects due solely to relative abundance. These properties make C. hyalina a good model organism for investigating sperm competition and female choice under laboratory conditions. In adition, molecular techniques can be used to characterize inbreeding (Packer and Pusey, 1993). and to identify parents, thus discriminating among potential parents

The aim of the present study was to investigate, under laboratory conditions, sperm competition and female choice in C. hyalina by exposing virgin colonies to sperm

suspensions made of sexually compatible allosperm, offered simultaneously in similar proportions. The offspring were genetically characterized to identify the proportion fathered by each allosperm donor. If female choice does not occur, the proportion of the progeny fathered by each allosperm donor should reflect the proportion of allosperm in suspension contributed by each donor. However, if females discriminate between males using a criterion that correlates with the male reproductive success or number of surviving offspring, it would be expected that some males will father a disproportionate number of progeny.

5.2 Material and methods

5.2.1 Clones, sperm suspensions and rearing conditions

Eleven clones of C. hyalina were used in the present study (A_1, D_1, E_1, F_1 J_1, $M_{1,}$, Q_1, A_2, QD_2 and QJ_9). Clones were originated, propagated and reared in reproductive isolation using standard methods (see Appendix 1). Allosperm suspensions were obtained using the dark-light reaction described in Appendix 2. Moreover, exposure to allosperm took place only when sperm concentration was sufficient not to limit fertilization (see Appendix 2). Experimental allosperm suspensions bearing similar concentrations of sperm from each donor (hereafter allosperm cocktail) were prepared by dilution with 0.2 µm filtered UV-treated seawater (hereafter FSW) to dilute. In all the experiments, 2-3 day old FSW was used.

5.2.2 Clone compatibility

In preliminary trials carried out 4 months before the onset of the experiment, 3 ramets of C. hyalina from each of the potential receptor clones were exposed separately to similar concentrations of sperm from the three allosperm donors. An extra ramet was maintained in reproductive isolation as a control for self-fertilization. The colonies were maintained in standard culture in 300 ml flat-bottomed glass vessels under standard culture conditions (see Appendix 1) until the presence of larvae in the brood chamber was noticed. The experimental vessels were then fitted with conditioned settlement units in order to assess if the larvae were able to produce viable ancestrulae. The settlement units were made of from conditioned acetate sheet that had been maintained in running seawater for at least three months (see Chapter 3). Viable ancestrulae were considered those able to metamorphose and survive for three weeks. From the preliminary trials, allosperm receptor clones were chosen to be D_1, H_1, F_1, J_1, Q_1, QJ_9, QD_2 and A_2, and clones A_1, M_1 and E_1 were chosen as allosperm receptor clones.

5.2.3 Experiment 1

The first experiment was carried out during November 1998 and January 1999. Colonies from each of 8 different clones were used. Ramets of clones D_1, H_1, F_1, J_1, Q_1, QJ_9, QD_2 and A_2 were used as allosperm receptors. Ramets of clones A_1, M_1 and E_1 were used as allosperm donors. All colonies had been maintained in reproductive isolation prior to the

experiments. During this period, no embryos or settled progeny were observed. To avoid risk associated with massive degeneration in the experimental colonies, two receptor colonies per clone were used. These two ramets were randomly removed from the mass culture bottles and assigned to two separate experimental vessels similar to those described in the previous section. In order to acclimatize them to the new conditions, the receptor colonies were maintained in the vessels for one week before exposure to allosperm. During this acclimatization period the colonies were cleaned and the water changed every day. The first attempt to obtain allosperm suspensions from the donor clones was begun only after acclimatization. Receptor colonies were allowed to stay in the allosperm cocktail for two hours and then washed with FSW for 5 minutes before being assigned to the experimental culture vessels. A third ramet of each clone was not exposed to the allosperm cocktail, but maintained in FSW under reproductive isolation to control for self-fertilization.

One ramet from each of the 3 clones used as allosperm donors was also used as a sperm receptor and exposed to a sperm cocktail including similar concentrations of each of the donor clones. A fourth ramet from each donor clone was maintained in reproductive isolation to control for self-fertilization.

5.2.4 Experiment 2

The second experiment was carried out from January to April 1999. In this second experiment colonies from each of 5 clones were used. Clones D_1, F_1, H_1, J_1, and QD_2 were chosen as allosperm receptors. The allosperm were the same as used in the previous experiments (i.e. A_1, M_1 and E_1). All colonies were assigned to the experimental vessels to acclimatize for two weeks before allosperm triggering was attempted. From each clone one ramet was exposed to 200 ml of allosperm cocktail consisting of similar concentrations of the three sperm suspensions. Moreover, one ramet from each of the 5 clones was separately exposed to 200 ml of suspension from each donor. This protocol enabled the compatibility between the clones to be evaluated simultaneously. As a control for self-fertilization, one ramet of each clone was maintained in reproductive isolation until the end of the experimental period.

5.2.5 Experiment 3

Ramets of the allosperm donor used in the Experiments 1 and 2 (i.e. A_1, M_1 and E_1) were used as sperm donors and exposed to a sperm cocktail including sperm from the same clones. One ramet from each donor clone was exposed to a triple cocktail including sperm from the three clones. A second ramet was exposed to a double cocktail in which autosperm was not present. Since self-fertilization is rare in C. hyalina, progeny in those colonies was expected to be fathered by allosperm. Four extra ramets were used, one ramet being exposed to a single sperm suspension from each donor. Thus, one ramet was exposed to autosperm and the other two to single allosperm suspensions. Finally, a ramet of each donor clone was maintained in reproductive isolation as a control for self-fertilization

5.2.6 Collection of progeny and experimental measurements

In both experimental runs, all receptor colonies were drawn using a camera lucida and a stereo microscope (Wild M4), between one and two days before they were exposed to allosperm. Drawings included the natural growing edge of each colony and the position of every visible female zooid. The drawings were used to assess if the progeny were produced in brooding chambers present inside the growing edge of the colonies at the time exposure to allosperm took place or beyond this margin.

To obtain larval settlement, culture vessels were equipped with two conditioned acetate sheets. Conditioned sheets had been maintained in running seawater for at least three months. One acetate sheet covered the bottom of the vessel and the other wrapped round the inner wall. Embryos were considered to be mature when their surface bore a ciliated corona (DJ Hughes, 1987). Once mature embryos were visible in the ovicells, the acetates were observed daily. The position and timing of settlement was noted in drawings of each settlement unit per vessel. In the first experiment, despite two ramets of each clone being used, sampling of material for DNA extraction involved only one colony. If one ramet died during the experiment, progeny was sampled from the surviving colony. However, if both colonies were alive when sampling took place, a sample was taken only from one

colony. When massive degeneration took place in both colonies, progeny were sampled from the less affected colony. In the second experiment, samples were taken from the colonies exposed to the allosperm cocktail and from the receptor colonies exposed to individual allosperm suspensions. DNA samples were taken from 3-4 zooids removed with the aid of a sterilised scalpel from the progeny 3 wk after settlement. The samples were stored in 1.5 ml micro tubes filled with 10 µl of TE buffer (10 mM Tris, 1mM EDTA) pH 7, and the DNA was extracted from the zooids between 20-30 minutes after the samples were taken. The DNA extraction was carried out by deep freezing the samples for 1 min in a bath of IMS at -80°C. Afterwards, the samples were boiled for 1 minute at 100 °C and again frozen by keeping the samples for 1 min at -80°C. For long-term storage, the micro tubes bearing the samples were subsequently transferred and maintained in a freezer at -80°C until the fingerprint analysis was done. Fingerprint analyses to assess paternity were conducted by M Richard and N. Hardman using polymorphic microsatellite markers developed for C. hyalina (Hoare et al., 1998). Procedures for polymerase chain reaction (PCR) amplification and genotying of individuals at one microsatellite loci were modified from Hoare (Hoare et al., unpublished).

In Experiment 1, samples for fingerprinting involved a representative proportion of progeny produced by the 8 colonies. 120 samples were collected from each receptor colony. Samples included zooids from the colonies produced by the first and last 30 settled larvae. Moreover, samples of zooids were collected from 60 colonies produced by larvae settling between these times. From the progeny produced by donor clones exposed the allosperm cocktail, only 30 random samples were taken. However, these samples were late embryos removed from the ovicells. A clean, sterilized needle was used to carefully crack the ovicells and the embryos were removed by suction using a Gilson® pipette. To avoid trapping embryos on the walls of the pipette tip, large diameter tips were used. Collected embryos were placed into wells of a multiwell plate and then placed in Eppendorf micro-tubes with 10 µl of TE (10 mM Tris, 1mM EDTA), pH=7. DNA extraction and microsatellite analysis on these samples were carried out as described above.

In Experiment 2, 110 samples were taken from the progeny of each receptor colony, including zooids from the first and last 40-settled ancestrulae and a random sample of zooids from 30 ancestrulae settled in between. In Experiment 3, from the progeny

produced by donor ramets exposed to the triple and double sperm cocktails, zooids from 20 colonies were taken as samples. However, from colonies exposed to single allosperm suspensions only 5 samples consisting of 3-4 zooids were taken from the newly settled colonies.

5.3 Results

5.3.1 Experiment 1

Embryos and larval settlement were found only in the colonies exposed to the allosperm cocktail. Moreover, in the control colonies neither embryos nor larvae were observed. Since all the receptor colonies were similar in size and in the proportion of active zooids when exposed to allosperm, the number of offspring produced for each receptor colony was roughly similar (Table 1). However, as a consequence of a massive degeneration of unknown origin in zooids of the clone A_2, the number of progeny was significantly reduced (Table 1).

In all the receptor clones, the embryos were concentrated in ovicells placed on top of active autozooids, mainly toward the growing edge of the colonies. The highest proportion of the progeny (ca. >0.82) was brooded in ovicells located within the colonial growing edge at the moment that exposure to allosperm took place. The rest of the progeny were brooded in ovicells located beyond this margin (Table 1). The proportion of the offspring surviving to the three weeks old stage exceeded 0.90 (Table 1).

Newly settled ancestrulae were found from day 16 to 41. Fingerprinting was possible for 856 of 858 samples. Fingerprinting showed that the progeny were the product of outcrossing, being fathered by the 3 allosperm donors present in the cocktail (Fig. 1). Only progeny from one colony was attributed to self-fertilization (Fig. 1). This corresponded to a few embryos produced by receptor clone A_2. On average, a high percentage of the progeny was fathered by the clone M_1 (ca. 50 %). However, on average similar percentages (range = 20-25 %) of the progeny were fathered by clones A_1 and E_1. With the exception of receptor colonies from clones D_1 and A_2, allosperm from M_1 fathered the highest percentage of the progeny (Fig. 1).

Fingerprints of progeny produced by ramets of sperm donors exposed to the triple allosperm cocktail showed that self fertilization was not responsible of the progeny produced by clones A_1 and M_1 (Fig. 2). However, 4 colonies produced by the clone E_1 (ca. 13 % of the sampled progeny) were found to be the result of self-fertilization.

Analysis of the first and last 30 settled ancestrulae show that they were fathered by the three allosperm donors represented in the cocktails (Table 2). On average, a high proportion (ca. > 0.55) of the first and last 30 ancestrulae was fathered by sperm from clone M_1. The rest of the progeny were fathered by allosperm from A_1 and E_1. Similarly, analysis of the ancestrulae settled between the first and last batches showed that a high proportion of the progeny was fathered by M_1 (Table 2). A proportion between 0.22 and 0.29 of the progeny was fathered by A_1 and E_1.

5.3.2 Experiment 2

As in the Experiment 1, embryos and larval settlement were only found in the colonies exposed to the allosperm cocktail. Again, in the control colonies of the receptor and donor clones neither embryos nor larvae were observed.

The number of offspring produced for each receptor colony was roughly similar (Table 3). In all the receptor clones the embryos were concentrated in ovicells placed on top of active autozooids, mainly toward the growing edge of the colonies. The highest proportion of the progeny (ca. 0.88) was brooded in ovicells located within the colonial growing edge at the moment that exposure to allosperm took place. The rest of the progeny were brooded in ovicells located beyond this margin (Table 3). The proportion of embryos becoming a three week old colony always exceeded 0.90 (Table 3).

Newly settled ancestrulae were found from day 18 to day 43. Fingerprint analysis of the first and last 40 settled ancestrulae showed that they were fathered by the three allosperm present in the cocktails (Table 4). From the 550 samples of progeny produced by receptor colonies exposed to allosperm cocktail, the fingerprint analysis was interpretable in only 447 cases. The analysis showed that all the progeny were the product of outcrossing, being fathered for by the 3 allosperm donors represented in the cocktails. No progeny were attributed to self fertilization (Fig. 3). On average, approximately one third (range = 30-37%) of the progeny was fathered by each allosperm donor represented in the allosperm cocktails. Analysis of the first and last settled ancestrulae showed that approximately one third of the progeny were fathered by each allosperm donor (Table 4). Similarly, analysis of the ancestrulae settled between them showed one third of the progeny were fathered in

similar proportions by each allosperm donor (Table 4).

A summary of the progeny produced by the receptor colonies exposed to individual allosperm suspensions is shown in Table 5. Most of the progeny (ca. 0.88) were produced by ovicells placed within the initial colonial growing edge. The rest of the progeny was produced by ovicells budded beyond this margin. A high proportion of embryos (ca. 0.85) were able to survive for 3 wk after settlement. Fingerprint analysis showed that all progeny were fathered by the corresponding allosperm donor; no self fertilization was found (Table 5).

5.3.3 Experiment 3

Fingerprint analysis for the progeny produced by the sperm donor clones exposed to a sperm cocktail are summarized in the Figure 4. When the colonies were exposed to the triple sperm cocktail, including autosperm (i.e. A_1, M_1 and E_1), the progeny were found be the product of outcrossing by the 2 allosperm donors represented in the cocktail. Similar proportions were fathered by each allosperm donor. No progeny were found to be the product of selfing (Fig 4, top graphs). A similar result was found when the colonies were exposed to double cocktails, including only allosperm. All the progeny were found be the result of outcrossing, without evidence of self fertilization (Fig 4, second row of graphs from the top). As in the triple cocktail, each allosperm donor fathered similar proportions of the progeny. Finally, when colonies were exposed to individual sperm suspensions, progeny were produced only when allosperm were used. Progeny were not produced by colonies exposed to autosperm suspensions (Fig. 4, last three rows of graphs) nor by colonies not exposed to autosperm suspensions but maintained in reproductive isolation (not included in Fig. 4). However, when colonies were exposed to allosperm suspensions all the progeny were products of outcrossing, being fathered by the corresponding allosperm donors. Selfed progeny were not found in these colonies (Fig 4, last three rows of graphs).

5.4 Discussion

5.4.1 Experiments 1 and 2:

The timing for the first and last settled ancestrulae in Experiments 1 and 2 are in agreement with previous evaluations of the brooding cycle in C. hyalina (see Chapter 3). The complete brooding cycle under laboratory conditions in C. hyalina, from the appearance of the oocytes in the female coelom to larval release took about 2-3 wk.

Production of progeny by female zooids located beyond the growing edge of the colonies when sperm exposure took place is in agreement with other findings in this dissertation (see Chapter 3). Since most of the progeny in the present study were the result of outcrossing (above), this proves that sperm storage and intracolonial sperm translocation took place.

The results of the present study show the prevalence of outcrossing in C. hyalina. Analysis of microsatellite showed that progeny were fathered mainly by the potential allosperm mates and not by autosperm. In Experiment 1, the only products of self fertilization originated from clone A_2. Most of the autoozoids in the ramets of this clone degenerated by the end of the first week, when most of the progeny were aborted. This suggests, that under conditions of stress such those producing degeneration, self-fertilization may be possible. However, this result does not agree with experiments specifically designed to investigate the possibility of self-fertilization under stressful conditions (see Chapter 7). In those experiments, colonies forcefully exposed to autosperm or colonies maintained in reproductive isolation under standard and stressful conditions did not produce progeny. This could be attributed to different effects on the putative mechanism stopping self fertilization between manipulated stress (i.e. deprivation of food and subjection to thermal shock) and stress producing massive degeneration in colonies of the clone A_2. In Experiment 2, no progeny were attributed to self-fertilization and none were produced by the control colonies. The only possible explanation of differences between Experiment 1 and 2 in connection with self fertilization are unknown effects of stress on the mechanism of controlling paternity. Massive degeneration of autozooids did not occur in Experiment 2. Evidence of self-fertilization in the colony of clone E_1 exposed to allosperm cocktail in Experiment 1 (Fig. 2) cannot be attributed to massive degeneration. This suggests that

exposure to high concentration of autosperm in the presence of allosperm could be overwhelm the mechanism for controlling paternity and avoiding self fertilization. Similar results, showing presence of a few progeny produced by self-fertilization under forced exposure to autosperm, are described in the Chapter 3. It seems, therefore that under certain circumstances self fertilization could be possible in C. hyalina. Since in Experiment 1. progeny from control colonies were removed from the ovicells as late embryos, maternal contamination in the samples could have taken place. However, the presence of only the maternal alleles at the scored locus without a third paternal allele in the same gel track suggests that this progeny was the product of self-fertilization. Moreover, alleles were not shared between the maternal and paternal colonies. This strongly suggests that self-fertilization occurred. However, since these embryos were collected before settlement, the viability or capacity of the embryos to complete the brooding cycle and survive for 3 wk was not assessed. Therefore, if self-fertilization did indeed take place, deleterious effects on embryo survival were not apparent. Inbreeding and outbreeding can both compromise fitness (Lynch, 1991). In C. hyalina, low survival of progeny attributed to self fertilization has been previously suggested as evidence of inbreeding depression (Hunter and Hughes, 1993c). The possibility of contamination during reproductive isolation by exogenous allosperm that shared alleles with the maternal clone cannot be discharged.

All allosperm donors were represented in the progeny produced by all the receptor colonies in Experiments 1 and 2. This suggests sperm mixing with acceptance of all the allosperm donors by the receptor colonies. This is in agreement with preliminary trials where individual sexual compatibility among the clones was demonstrated. In Experiment 1, despite allosperm being present at similar and non-limiting concentrations, clone M1 produced more progeny. This could be the consequence of active selection for this allosperm by the receptor colonies or by an unknown mechanism, which allowed allosperm from clone M_1 to overcome the other two allosperm. Since all allosperm in the cocktail were released from the donors simultaneously, this result cannot be attributed to sperm ageing. In Experiment 1 and 2, the number of progeny produced by colonies exposed to allosperm cocktail (Table 1) was similar to the number of progeny produced by similar sized ramets of the same clones exposed to sperm cocktail in Experiment 2 (Fig. 2). It therefore seems reasonable

to conclude that the number of available eggs in the receptor colonies was limiting, and that competition between allosperm present in the cocktail occurred. Since clone M_1 fathered the highest percentage of the progeny in Experiment 1 (Fig. 1), it may be concluded that there was either differential sperm performance under competition or selective female choice. However, progeny fathered in similar percentages by each one of the sperm donors in the Experiment 2 (Fig. 3), suggests the absence of female choice. Considering that differential mortality could take place in brooded embryos, results of Experiment 1 can be interpreted as due to the result of abortion of progeny fathered by clones A_1 and E_1. However, evidence of abortion of growing oocytes was not recorded. The results of fingerprint analysis, together with settlement timing, indicate that all allosperm were used in similar proportions throughout the settlement period, showing that sperm mixing took place during the storage.

5.4.2 Experiment 3

Absence of progeny attributed to self fertilization from colonies exposed to triple cocktails (Fig. 3), where autosperm was present, is not in agreement with the results of Experiment 1 (Fig. 2). This could be explained by self-fertilization taking place when colonies are under stress, as was the case for some days during the Experiment 1. However, experiments specially designed to test the possibility of self fertilization under manipulated stress conditions failed to find progeny in those colonies. The absence of progeny produced by receptor colonies exposed to double cocktails is in agreement with the findings for the triple cocktails. Since in both situations progeny were fathered in similar proportions to the allosperm, these results suggest allosperm mixing occurred during the storage and that both allosperm were chosen about equally.

Absence of progeny produced by self-fertilization when single allosperm exposures were used and in the control colonies maintained in reproductive isolation suggests that the female mechanism stopping self-fertilization works similarly in both situations. The results of the present study show prevalence of outcrossing in C. hyalina. This is in agreement with results of previous chapters in this dissertation. Moreover, the present supports previous results, indicating intracolonial sperm movement in C. hyalina (see Chapter 3). Fingerprint analysis suggests that receptor colonies are using all the sexually

compatible sperm offered in the cocktails. This suggests a lack of selective female choice for a particular sperm or sperm competition i.e. where one particular sperm overcomes the rest. Evidently, therefore, all the allosperm were either similarly "attractive" to the females or the "quality" of the allosperm used in the experiments was similar. Thus, allosperm were used by females in the available proportions and no disproportionate usage through sperm competition took place. The absence of preference by female zooids in C. hyalina (i.e. female choice) could be an adaptive response to maximize sperm usage. This could be an important strategy in natural conditions if sperm are diluted and sperm capture dependent on active mechanisms in the receptor colonies.

In the present experiment all the allosperm used in the cocktail were supposedly unrelated and sexually compatible with the receptor colonies. Moreover, similar numbers of progeny were produced by colonies exposed to single suspensions and sperm cocktails. However, since fewer progeny have been found when receptor colonies were exposed to sperm suspensions of mother full-sib clones than in receptor colonies exposed to more unrelated sperm, i.e. maternal half-sib or totally unrelated (see Chapter 7), different results can be found. Either some sperm could be preventing progeny being fathered mainly by the "chosen" or "winning" sperm. At the time this dissertation was being produced, two experiments of sperm competition and female choice relative to coancestry (the degree in which sperm donors and sperm receptors are genetically related, Michod, 1993) had been unsuccessfully done. As a consequence of massive degeneration in the receptor colonies, most of the progeny were lost. The third attempt to test the effect of coancestry on sperm competition or female choice in C. hyalina was in progress when this chapter was written. Since self fertilization is not common in C. hyalina, female active discrimination as mechanism of allosperm recognition and sperm competition may play an important role when mating involve genetically related colonies. To date, the relative role of female choice and sperm competition in determining paternity in C. hyalina remains speculative. Sperm adaptation for sperm competition could work if receptor colonies allow access to their sperm stores. Evidence in Chapter 2, showed that a receptor colony can store sperm up to 4-5 weeks, and therefore fertilization via sperm competition remains a possibility. Since in the present study the receptor colonies were exposed to allosperm suspensions simultaneously,

differential position close to the area leading to the eggs could not encourage a first-in / first out bias. The relative role of sperm competition and female choice determining fertilization success cannot be fully understood without knowledge of both the anatomical or physiological mechanisms involved in sperm storage. Therefore, futures studies should incorporate this theme (e.g. Bishop and Sommerfedt, 1996). At the moment this thesis was written an experiment to identify the location of allosperm storage in colonies of C. hyalina, using radio-labelled sperm, was still in progress (November, 1999).

Table 1: Experiment 1. Number of embryos and viable ancestrulae produced by each receptor ramet of C. hvalina exposed simultaneously to an allosperm cocktail (i.e. sperm from clones A_1, M_1 and E_1). The first three columns represent the total amount of progeny (i.e. embryos) and the number of embryos brooded inside and beyond the growing edge of the colonies at the moment that allosperm exposure took place. The number in parentheses represents the percentage of embryos corresponding to each category as a proportion of the total. Viable ancestrulae correspond to the number of ancestrules alive 3 wk after settlement. The number in parentheses corresponds to the number of newly settled colonies from which samples were taken for fingerprint analysis. S = proportion of embryos becoming a viable ancestrula.

	Progeny			viable	S
Receptor clone	Total	Inside	Beyond	ancestrulae	
D_1	177	155 (0.88)	22 (0.12)	168 (120)	0.95
F_1	142	124 (0.87)	18 (0.13)	137 (120)	0.97
H_1	190	157 (0.82)	33 (0.17)	173 (120)	0.91
J_1	166	141 (0.85)	25 (0.15)	154 (120)	0.93
Q_1	157	143 (0.91)	14 (0.09)	152 (120)	0.97
QD_2	144	140 (0.97)	4 (0.03)	141 (120)	0.98
QJ_9	173	161 (0.93)	12 (0.07)	158 (120)	0.91
A_2	19	18 (0.95)	1 (0.05)	18 (18)	1.00

Table 2: Experiment 1. Temporal proportion of progeny produced by each receptor colony being fathered by each of the 3 different allosperm present in the suspensions (A_1, M_1 and E_1). Analysis included the first 30 settled ancestrulae, the last 30 settled ancestrulae and 60 random ancestrulae settled between both batches of larvae. R.C = receptor clone. Note, since massive degeneration occurred in clone A_2, only 19 ancestrulae were produced and so are not included here.

	First 30 settled ancestrulae			Sixty random ancestrulae settled in between			Last 30 settled ancestrulae		
R.C	A_1	M_1	E_1	A_1	M_1	E_1	A_1	M_1	E_1
D_1	0.50	0.40	0.10	0.38	0.40	0.20	0.40	0.43	0.17
F_1	0.30	0.53	0.17	0.20	0.70	0.10	0.17	0.63	0.20
H_1	0.37	0.53	0.10	0.17	0.70	0.13	0.23	0.68	0.09
J_1	0.27	0.43	0.30	0.30	0.57	0.13	0.19	0.54	0.27
Q_1	0.27	0.70	0.03	0.27	0.50	0.23	0.15	0.48	0.37
QD_2	0.40	0.47	0.13	0.32	0.58	0.10	0.45	0.55	0
QJ_9	0.30	0.63	0.07	0.23	0.57	0.20	0.08	0.62	0.30
mean	0.34	0.53	0.13	0.29	0.57	0.14	0.22	0.56	0.22
(SE)	0.03	0.04	0.03	0.04	0.04	0.03	0.04	0.04	0.04

Table 3: Experiment 2. Number of embryos and viable ancestrulae produced by each receptor ramet of C. hyalina exposed simultaneously to an allosperm cocktail (i.e. sperm from clones A_1, M_1 and E_1). The three first columns represent the total amount of progeny (i.e. embryos) and the number of embryos brooded inside and beyond the growing edge of the colonies at the moment that allosperm exposure took place. The number in parentheses represents the percentage of embryos corresponding to each category as a proportion of the total. Viable ancestrulae correspond to the number of new colonies alive 3 wk after settlement. The number in parentheses corresponds to the number of newly settled colonies from which samples were taken for fingerprint analysis. S = proportion of embryos becoming a viable ancestrula.

Receptor clone	Progeny			viable ancestrulae	S
	Total	Inside	Beyond		
D_1	147	135 (0.92)	12 (0.08)	137 (110)	0.93
F_1	123	115 (0.94)	8 (0.06)	121 (110)	0.98
H_1	223	197 (0.88)	26 (0.12)	201 (110)	0.90
J_1	172	166 (0.97)	6 (0.03)	154 (110)	0.90
Q_1	236	219 (0.93)	17 (0.07)	217 (110)	0.92

Table 4: Experiment 2. Temporal proportion of progeny produced by each receptor colony being fathered by each of the 3 different allosperm present in the allosperm suspensions (A_1, M_1 and E_1). Analysis included the first 40 settled ancestrulae, the last 40 settled ancestrulae and 30 random ancestrulae settled between both batches of larvae. R.C = receptor clone.

	First 40 settled ancestrulae			Thirty random ancestrulae settled in between			Last 40 settled ancestrulae		
R.C	A_1	M_1	E_1	A_1	M_1	E_1	A_1	M_1	E_1
D_1	0.43	0.33	0.24	0.30	0.33	0.37	0.34	0.33	0.33
F_1	0.45	0.35	0.25	0.40	0.23	0.37	0.33	0.27	0.40
H_1	0.41	0.28	0.31	0.37	0.43	0.20	0.40	0.18	0.42
J_1	0.33	0.42	0.25	0.30	0.37	0.33	0.43	0.23	0.35
QJ_9	0.28	0.38	0.33	0.53	0.17	0.30	0.36	0.21	0.43
mean (SE)	0.37 (0.03)	0.35 (0.02)	0.28 (0.02)	0.28 (0.02)	0.31 (0.04)	0.31 (0.03)	0.37 (0.02)	0.24 (0.02)	0.39 (0.02)

Table 5: Experiment 2. Number of embryos and viable ancestrulae produced by each receptor ramet of C. hvalina exposed individually to A_1, M_1 and E_1 allosperm suspension. The first three columns represent the total amount of progeny (i.e. embryos) and the number of embryos brooded inside and beyond the growing edge of the colonies at the moment that allosperm exposure took place. The number in parentheses represents the percentage of embryos corresponding to each category as a proportion of the total. Viable ancestrulae correspond to the number of new colonies alive 3 wk after settlement. The number in parentheses corresponds to the number of newly settled colonies from which samples were taken for fingerprint analysis. S = the proportion of embryos becoming a viable ancestrula and PF = the proportion of the progeny fathered by the clone used as allosperm donor.

Allosperm suspension A1:

Receptor clone	Progeny			viable ancestrulae	S	PF
	Total	Inside	Beyond			
D_1	168	157 (0.93)	12 (0.07)	157 (10)	0.95	1
F_1	139	125 (0.90)	14 (0.10)	135 (10)	0.97	1
H_1	199	195 (0.98)	4 (0.02)	188 (10)	0.95	1
J_1	162	158 (0.98)	4 (0.02)	160 (10)	0.99	1
QJ_9	210	201 (0.96)	9 (0.04)	208 (10)	0.99	1

Allosperm suspension M_1:

Receptor clone	Progeny			viable ancestrulae	S	PF
	Total	Inside	Beyond			
D_1	197	187 (0.92)	10 (0.08)	178 (10)	0.90	1
F_1	98	94 (0.94)	4 (0.06)	95 (10)	0.97	1
H_1	246	236 (0.88)	10 (0.12)	210 (10)	0.85	1
J_1	194	188 (0.97)	6 (0.03)	154 (10)	0.90	1
QJ_9	236	219 (0.93)	17 (0.07)	217 (10)	0.92	1

Allosperm suspension E_1:

Receptor clone	Progeny			Viable ancestrulae	S	PF
	Total	Inside	Beyond			
D_1	124	123 (0.99)	1 (0.01)	118 (10)	0.95	1
F_1	143	135 (0.94)	8 (0.06)	121 (10)	0.85	1
H_1	177	172 (0.97)	5 (0.03)	170 (10)	0.96	1
J_1	152	166 (0.97)	6 (0.03)	146 (10)	0.96	1
QJ_9	209	201 (0.96)	8 (0.04)	200 (10)	0.96	1

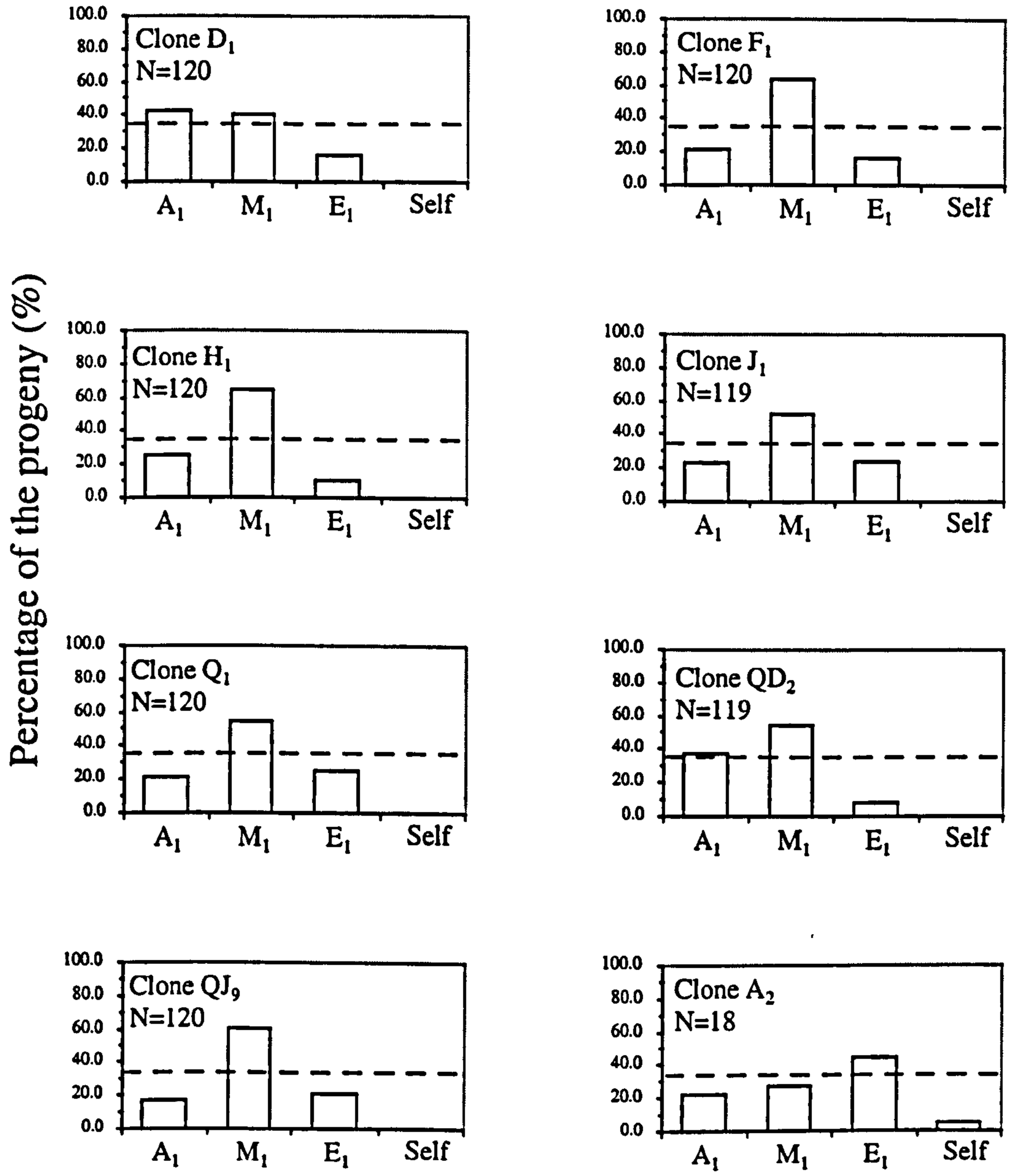

Figure 1: Experiment 1. Percentage of the sampled progeny from each of 8 clones used as receptor colonies fathered by the each of 3 clones used in the allosperm cocktail (i.e. A_1, M_1 and E_1). N = number of progeny analysed. Dashed lines correspond to 33% of the progeny.

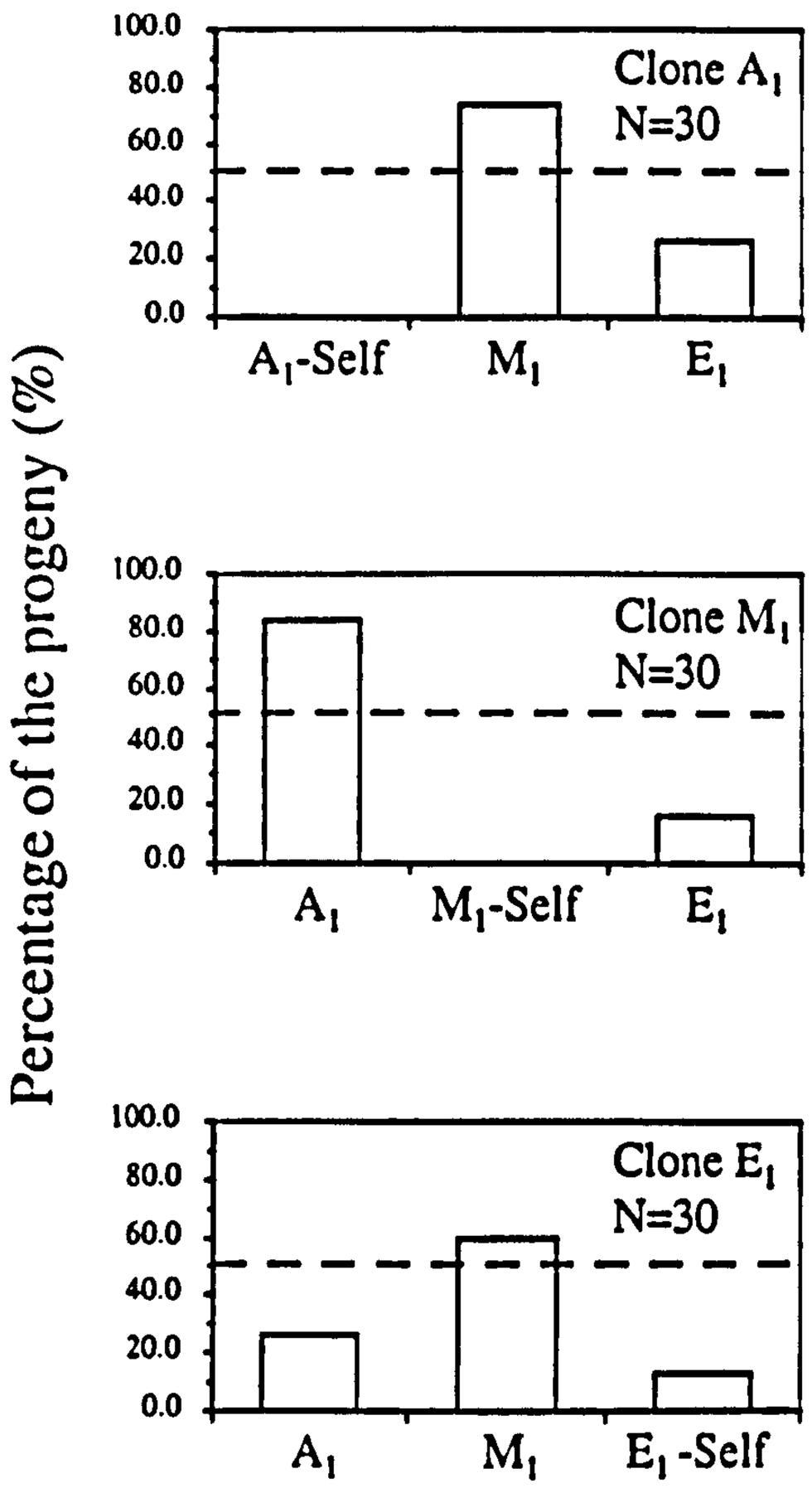

Figure 2: Experiment 1. Percentage of the sampled progeny from each of 3 clones used as allosperm donors fathered by the each of 3 clones used in the allosperm cocktail (i.e. A_1, M_1 and E_1). N = number of progeny analysed. Dashed lines correspond to 50 % of the progeny.

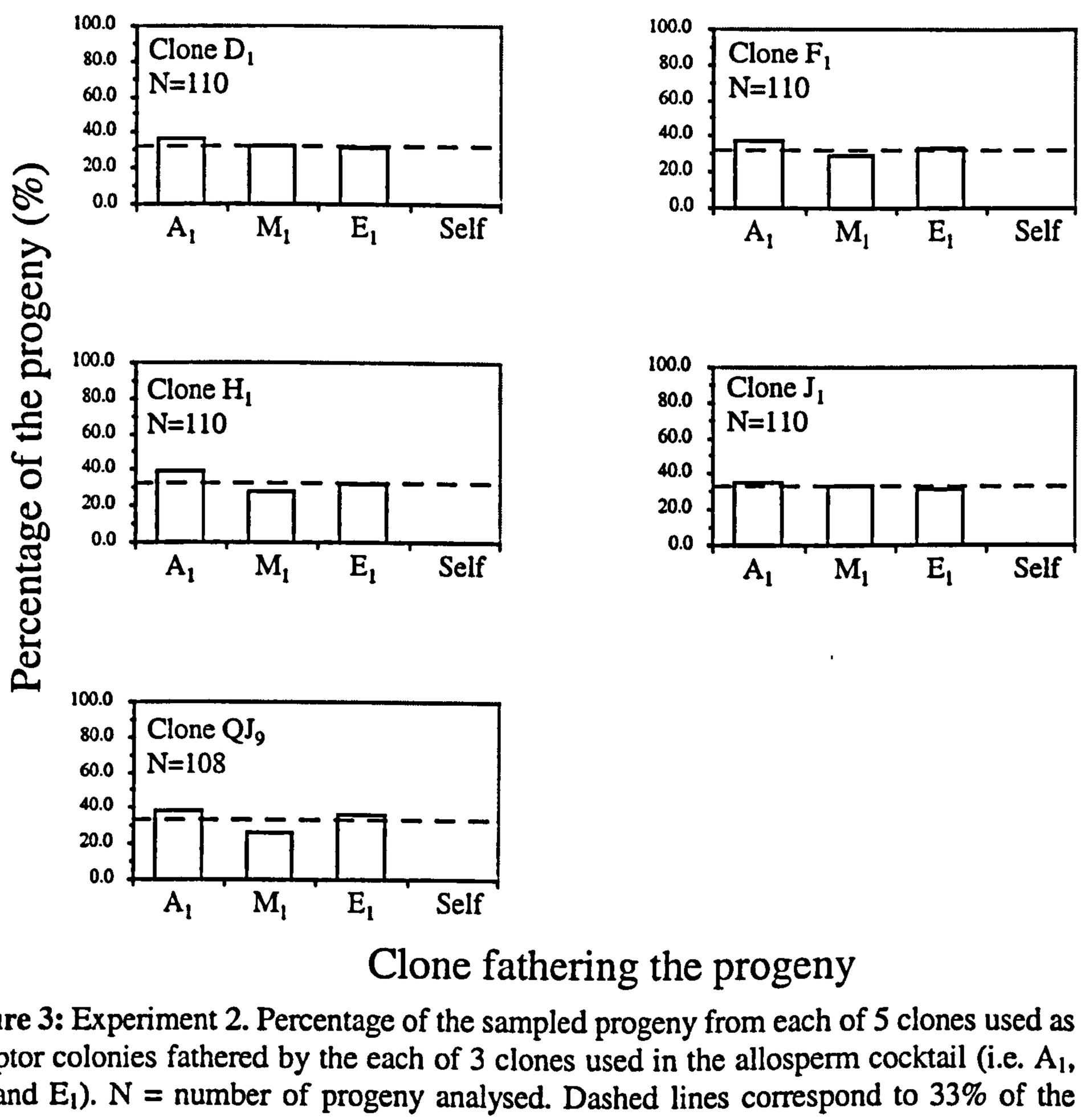

Figure 3: Experiment 2. Percentage of the sampled progeny from each of 5 clones used as receptor colonies fathered by the each of 3 clones used in the allosperm cocktail (i.e. A_1, M_1 and E_1). N = number of progeny analysed. Dashed lines correspond to 33% of the progeny.

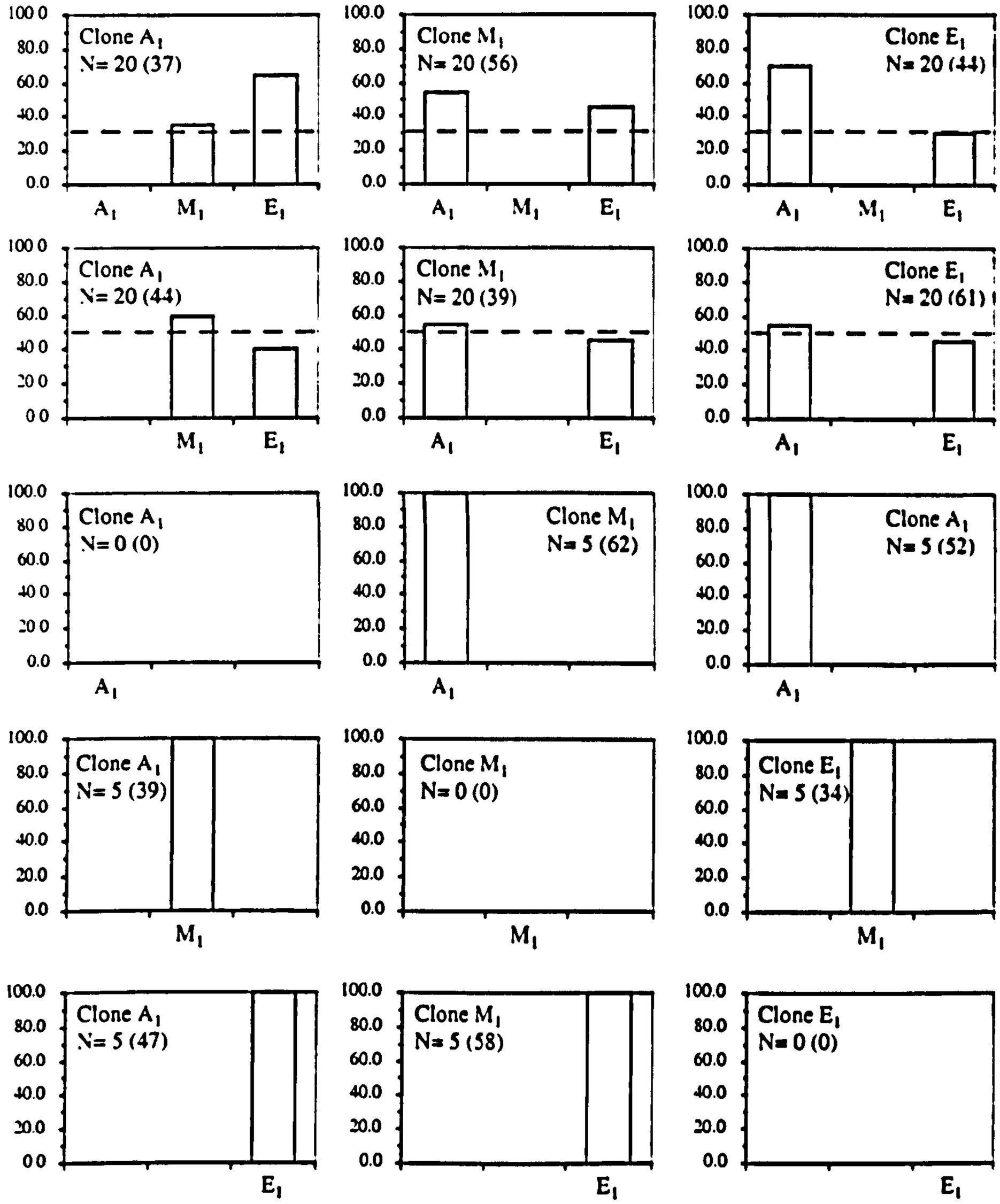

Figure 4: Experiment 3. Percentage of the sampled progeny from each of 3 clones previously used as allosperm donors but now used as sperm receptors. One ramet of each clone was exposed to an allosperm cocktail where sperm from clones A_1, M_1 and E_1 were present in similar concentrations (top row of graphs). A second ramet of each clone was exposed to an allosperm suspension where only two allosperm were present, again in similar concentrations. In this suspension, autosperm were not included (second rows of graphs from the top). Three extra ramets of each of 3 clones were exposed individually to sperm suspensions (the last 3 rows of graphs). One of them was exposed to autosperm suspension and the others to allosperm suspensions. N = number of samples used for fingerprinting; the number in parentheses corresponds to the total number of progeny produced by each receptor colony. Dashed lines in top graphs are 33% and 50% in the rest of the graphs.

Chapter 6:
Sperm precedence

6.1 Introduction

Hermaphroditic organisms possess a functional male and female during at least part of their lives (Michiels, 1998). As self-fertilization is possible in some hermaphrodites (see Michiels, 1998 for review), competition for fertilization opportunities may be expected between selfsperm and allosperm and between different allosperm. Colonies of the marine bryozoan C. hyalina are a good example of simultaneous hermaphroditism. Although colonies of this species are naturally exposed to both selfsperm (i.e. sperm produced by the colony itself) and allosperm (i.e. sperm produced by a genetically different colony), recent studies show that self-fertilization in laboratory reared colonies under strict reproductive isolation can occur (Cancino, 1983; Hunter and Hughes 1993c; Yund and McCartney, 1994, Hoare et al., 1999; this thesis). It can therefore be expected that if a receptor colony of C. hyalina receives sperm from different colonies, the allosperm may "compete" for fertilization opportunities. The degree to which sperm from one colony can fertilize available eggs in the receptor colony, at the expense of fertilization of eggs by sperm from other colonies, is termed sperm precedence. In species in which females mate more than once, competition between sperm from different males to fertilize the eggs has important implications for the mating strategies of both sexes (Parker 1970). Evidence of sperm competition in the animal kingdom occurs in a wide range of taxa (Birkhead and Parker, 1997). Studies on patterns of paternity, i.e. the proportion of the progeny fertilized by males in relation to their order of mating, have shown that the proportion of eggs fertilized by the second male to mate (termed P_2 by Boorman and Parker, 1976) can adopt different adaptive trends (see Birkhead and Parker, 1997). Thus is possible to find first male precedence (low values of P_2), last male precedence (high values of P_2) and absence of male precedence or mixing (values of P_2 close to 0.5) (see Parker, 1977).

The existence and relevance of sperm competition in C. hyalina has been reported by Yund and McCartney (1994). In their study, relatively less fertilizations were made by a male-functional colony when a second male-functional colony was maintained near the experimental female-functional colony. This result shows that in C. hyalina competition

between sperm from different males affects male reproductive success. The sperm competition theory predicts competitive interactions between different allosperm as a result of female effort to increase the quality of her offspring. However, a more active female role might be possible if females could exert some control over the paternity of their offspring. This could work in colonies of C. hyalina if, after insemination, sperm storage takes place. In this species vitellogenic oocytes have been observed for up to 40 days after insemination (see Chapter 3). If sperm storage takes place, sperm used in fertilization could be the result of sperm mixing, sperm precedence or female choice.

The present study aims to examine sperm use in relation to the order of mating (i.e. order of exposure to allosperm suspensions) in virgin colonies of C. hyalina. In a replicated experiment the dosage of two sexually compatible allosperm suspensions was manipulated to examine relative fertilization success. Moreover, the effects of the temporal gap between the two allosperm dosages on paternity were evaluated. The progeny were analyzed using DNA fingerprinting to assess paternity and potential patterns of sperm precedence. If sperm precedence does not occur, it would be expected that progeny will be fathered by both allosperm (sperm mixing), without the first dosed allosperm overcoming temporally the second one. However, if first-male or first-sperm precedence occurs, it would be expected that more progeny should be fathered by the first allosperm dosage. On the other hand, if last-male or last-sperm precedence occurs, more progeny should be fathered by the second allosperm dosage.

6.2 Material and methods

6.2.1 Clones, sperm suspensions and rearing conditions:

Clones (A_1, E_1, F_1, J_1, H_1, M_1, A_2 and QD_2) of the experimental organism, Celleporella hyalina (L.) were used. These clones were established, propagated and reared in reproductive isolation using the corresponding standard methods (see Appendix 1).

Allosperm suspensions were obtained using the dark-light reaction described in the Appendix 2. Exposure to allosperm took place only when sperm concentration was sufficient not to limit fertilization (see Appendix 2). Experimental allosperm suspension bearing similar concentrations of each donor (hereafter allosperm cocktail) was prepared using FSW to achieve similar final allosperm concentration. During the experiments the colonies were maintained under reproductive isolation using 300 ml flat-bottomed glass vessels under standard culture conditions (see Appendix 1). Two or three day old 0.2µm filtered and UV-treated seawater (hereafter FSW) was always used in the experiments. To obtain larval settlement , the same settlement units used in previous experiments of this thesis were used (see Chapter 2). The position and timing of settlement was noted in drawings of each settlement unit per vessel. Samples of progeny included zooids from the colonies produced by the first and last 20 settled larvae and zooids collected from random colonies produced by larvae settling between these times. DNA samples were obtained from 3-4 zooids removed with the aid of a sterilised scalpel from the new colonies 3 wk after settlement using the same protocol described in Chapter 5.

6.2.2 Clone compatibility

Preliminary trials, carried out 6 months before the onset of the experiment, assessed sexual compatibility between the potential clones of C. hyalina. Sexually compatible clones were considered as those able to produce viable progeny, i.e. progeny able to metamorphose and survive for three weeks. Two virgin ramets of each potential receptor clone were removed from reproductive isolation and exposed separately to allosperm suspensions of the potential donors. One colony was exposed to an allosperm suspension from clone A_1 and the other colony to an allosperm suspension from the clone M_1. The receptor colonies were exposed to 250 ml of roughly similar concentration of allosperm for 2 hours. During the time that the receptor colonies were

exposed to the allosperm suspension the vessel was kept in still water and dosed with 0.5 ml of R. reticulata suspensions. Under this condition, fertilization is more effective than in a system aerated and without food (own unpublished data).

Likewise two ramets from each receptor clone were used as a control and not exposed to allosperm suspensions. In order to assess if self-fertilization is possible when colonies are exposed to a solution of autosperm, one control ramet from each clone was exposed, at the beginning of the experiments, to autosperm for two hours. However the other ramets were removed from the PET bottles (see Appendix 1) and assigned in reproductive isolation to the experimental vessels. All the experimental colonies were maintained under the same conditions (see Appendix 1).

6.2.3 Experiment 1:

Healthy-looking, virgin C. hyalina ramets from the compatible receptor clones E_1, J_1, A_2 and QD_2 were haphazardly chosen and removed from the culture system, to be used as allosperm receptors. Healthy-looking colonies were considered to be those colonies bearing mainly active autozooids with a circular shape and without any sign of bearing embryos or larvae.

Six ramets from each sperm receptor clone were chosen. Two of those ramets were exposed first for two hours to 250 ml of allosperm suspension from clone A1, then carefully washed for 5 min with aged FSW before being exposed for two hours to 250 ml of the second allosperm suspensions from clone M_1. Using the same protocol, two more ramets were exposed first to allosperm suspensions from clone M_1 and then to allosperm suspensions from clone A_1. Once the colonies were exposed to the second allosperm suspension, they were carefully washed and assigned to the experimental vessels. The two remaining ramets were used as a control for self-fertilization. One ramet was exposed for two hours to an autosperm suspension, and the other maintained in reproductive isolation. This last colony was not forcefully exposed to a suspension of autosperm, however, since all the experimental colonies were already exposed to autosperm suspensions. In this controlled mating, the receptor colonies and the sperm suspensions were maintained following the same protocol described in the clone compatibility section.

Colonies were observed daily and the presence of the first and the last oocyte inside the female zooids noted in camera lucida drawings. The period between the allosperm dosage and the appearance of the last oocyte was considered as the maximum sperm storage.

6.2.4 Experiment 2:

The protocol in this experiment was the same as used in the previous experiment, except that ramets of clones F_1 were added to the clones H_1, J_1, A_2 and QD_2 used as sperm receptors in the experiment 1. Ramets of clones A_1 and M_1 were used as allosperm donors.

6.2.5 Experiment 3:

This experiment was carried out using ramets of the same clones used in the previous experiment (i.e. F_1, H_1, J_1, A_2 and QD_2 as allosperm receptor clones and, A_1 and M_1 as allosperm donor clones). However, from each clone 10 ramets were assigned to the following treatments in order to evaluate the effects of mating interval and mating sequence. Two mating intervals were tested, a shorter interval of 2 h between two consecutive exposures to allosperm suspensions (hereafter short interval) and, a longer interval of 48 hours (hereafter long interval). Moreover, when the two successive matings were 2 or 48 h apart the mating sequence was altered, i.e. in one experiment allosperm used as first allosperm donors were used in the other as second donors.

6.2.5.1 Part A: treatments where colonies were mated first with allosperm from clone A_1

Three ramets of each clone were exposed first to fresh sperm from clone A_1 for 2 hours and then removed and rinsed with aged FSW for three minutes. One of these ramets was chosen randomly and transferred to a culture vessel to serve as a control for sperm fertilization capacity under the experimental conditions (Controls for A_1, short interval). One of the remaining ramets was exposed to fresh sperm of M_1 for two hours, care having been taken to rinse off sperm from clone A_1. After this period, the ramet was rinsed with aged FSW for three minutes and transferred to a culture vessel. This ramet was considered as being exposed first to sperm from clone A_1 and then to sperm from

clone M_1 with a short interval between the two exposures (Treatment A_1 - M_1, short interval). In order to evaluate fertilization capacity of the sperm suspension, an extra ramet was exposed to a single suspension of allosperm from clone M_1 (Control for M_1, short interval). The third ramet was rinsed with FSW and maintained in reproductive isolation for 48 hours, then exposed to a fresh suspension of allosperm from clone M_1 for two hours (Treatment A_1 - M_1, long interval). As before, an extra ramet was exposed to a suspension of allosperm from clone M_1 to evaluate fertilization capacity of this sperm (Controls for M_1, long interval).

6.2.5.2 Part B: treatments where colonies were mated first with allosperm from clone M_1

Using the protocol described above, ramets of the receptor clones were exposed to the same allosperm suspensions but in the reverse direction. However, due to logistic difficulties associated with handling many experimental colonies without risk of contamination, this part of the experiment was started one day after the previous one. Therefore, different suspensions of the same allosperm were used in the two parts of the experiment. However, in all the cases sperm concentration was sufficient not to limit fertilization (see Appendix 2) and fresh sperm with maximum fertilization capacity was used. For each receptor clone, two virgin ramets were kept in reproductive isolation as a control for self-fertilization.

6.2.6 Experimental measurements

In all the experiments, colonies were drawn using a camera lucida and a stereo microscope (Wild M4), between one and two days before exposure to allosperm. Drawings included the natural growing edge of each colony and the position of every visible female zooid. The drawings were used to assess if the progeny were produced in brood chambers present inside the growing edge of the colonies at the time when exposure to allosperm took place, or beyond this margin. Number of basal males, frontal males, active and recycling autozooids were counted in all the colonies one day before exposure to allosperm and then again 4 weeks later. Counts included female zooids with a complete brood chamber (i.e. established females) and females with an incomplete brood chamber (i.e. incipient females). Active autozooids were considered to be those with a complete feeding

system. while those without were considered to be recycling zooids. From these counts, values of sex ratio (female zooids/male zooids) and sexual allocation (sexual zooids/active autozooids) were derived. All counts were made using 40 x 10 magnification and a 1200 x 1200 µm graticule placed beneath the experimental substratum.

Proportions of progeny fathered by the first or second allosperm donors (i.e. P_1 and P_2) were compared using paired-samples t test or ANOVA of arcsine square root-transformed values. Colony size, sex ratio and sexual allocation were respectively log, log+1 and arcsin square root transformed (Sokal and Rolf, 1995) and compared using ANOVA. All statistical analyses were conducted using SPSS 8.0 data analysis program.

6.3 Results

6.3.1 Preliminary experiments: Clone compatibility

Oocytes, embryos and newly settled progeny were recorded for all the experimental clones exposed to both sources of allosperm (Table 1). On the other hand, over the same period of time neither developing oocytes and nor newly settled progeny were recorded for the control colonies maintained in reproductive isolation (Table 1). As in previous experiments, more vitellogenic oocytes, embryos and larvae were produced by colonies bearing more active zooids. However, in the present experiment the total number of newly settled colonies produced by each receptor colony was roughly similar (Table 1).

6.3.2 Experiment 1:

6.3.2.1 Self-fertilization and brooding time

The appearance of the first and last oocytes in the receptor colonies is summarized in the Table 2. The first and last oocytes were visible within the coelom of female zooids from day 4 to 8 and from day 28 to 38 respectively (Table 2).

Presence of oocytes, embryos and production of larvae was recorded in clones J_1, A_2, and QD_2. However, oocytes, embryos and progeny were not produced in the clone E_1 (Table 3), nor in the control colonies of each clone maintained in reproductive isolation (Table 3). A significant number of developing oocytes and embryos were found in the clones J_1, A_2 and QD_2. These numbers are similar to those recorded in the clone compatibility experiment (above) when allosperm were dosed separately.

6.3.2.2 Sperm storage and intracolonial sperm movement

In all the colonies exposed to allosperm suspensions, a proportion of the progeny was produced by females present at the beginning of the experiment with fully developed brood chamber (ovicell), or by females without a fully developed brooding chamber (Table 4). Although most of the progeny were produced by females within the initial area of the colony, a small proportion of the embryos were produced by females budded beyond the original limits of colony and therefore which were not present when allosperm dosage took place (Table 4).

6.3.2.3 Fingerprint analysis and sperm precedence

As a consequence of massive degeneration causing lost of most of the progeny produced by the receptor, samples from this experiment were not considered.

6.3.3 Experiment 2:

6.3.3.1 Clone compatibility

See Table 1.

6.3.3.2 Brooding time and self-fertilization

The appearance of the first and last oocytes is summarized in the Table 8. The first and last oocytes were visible in the coelom of female zooids from day 2 to 7 and from day 25 to 39 respectively (Table 5). Progeny were produced by all the experimental colonies exposed to allosperm suspensions. On the other hand, progeny were not produced by the control colonies, and only one colony was considered the product of self-fertilization (Table 6). Values of fertilization success were high and similar (Table 6).

6.3.3.3 Sperm storage and intracolonial sperm movement

In all the experimental colonies exposed to allosperm suspension, most of the progeny was produced within the original colony margin (Table 7). However, some of the progeny was also found in ovicells budded beyond the original margin of the colony (Table 7).

6.3.3.4 Fingerprint analysis and sperm precedence

Fingerprint analysis did not show any evidence of contamination from extraneous sources. Nearly all the progeny analysed were the product of fertilization by the experimental allosperm suspensions. Only one isolated case of self-fertilization was found. However, a suspicious and anomalous trend, with much more progeny being fathered by allosperm M_1, was found (Table 6). This result occurred when allosperm from M_1 was the first or the second sperm suspension to which receptor colonies were exposed. Although massive abortion of embryos was not observed during the present experiment, extensive degeneration of autozooids was observed from the second

experimental week. Since differential mortalities of early, unobservable embryonic stages could have occurred, results of sperm precedence could be an experimental artefact and so are not considered in the conclusion section.

6.3.4 Experiment 3:

6.3.4.1 Colony compatibility

The results of sperm compatibility between the clones involved in the present experiment are those shown in the previous section, and are summarized in Tables 1. Progeny were produced in all the experimental clones exposed to allosperm suspensions from clone A_1 and M_1 (Table 1). Over the same period of time, oocytes and progeny were not found in the control colonies maintained in reproductive isolation (Table 1).

6.3.4.2 Brooding time and self-fertilization

The appearance of the first and last oocytes are summarized in the Table 8. The first and last oocytes were visible in the coelom of females zooids from day 3 to 7 and from day 27 to 41 respectively.

Embryos and viable progeny were found in all the colonies exposed to allosperm suspensions. These results were found in colonies exposed to a single allosperm suspension and in those colonies exposed to two allosperm suspensions. However, in control colonies not exposed to allosperm suspensions, embryos and progeny were not found. In fingerprint analyses of progeny produced by colonies exposed to two allosperm suspensions (Tables 9 and 10), it was possible to score 1134 of the 1160 samples (ca. 97.76%). In the rest of the samples, absence of maternal or other alleles at the scored locus in the corresponding gel track was found. Ambiguous scores, as those with only one clear maternal allele were also not included in the final scoring. 1132 scored samples (ca. 99.82 %) were found be the product of outcrossing with the allosperm donors. Only 2 colonies were scored as the product of self-fertilization (ca. 0.18 %).

In fingerprint analyses of progeny produced by colonies exposed to one allosperm suspension, it was possible to score 119 of the 120 samples (99.17%). These samples included progeny of ramets of clones F_1, H_1, A_2 and QD_2 exposed to separate and single

allosperm from A_1 or M_1. Progeny produced by ramets of clone J_1 exposed to single allosperm suspensions died after settlement and so were not collected for scoring. 114 scored samples (95.80%) were found to be the product of outcrossing by the experimental allosperm donors. In 5 samples, evidence of allosperm contamination was found (4.20%).

6.3.4.3 Sperm storage and intracolonial sperm movement

Progeny were produced within and beyond the initial growing edge present at the moment that allosperm took place. Within the initial growing edge, progeny were produced in completed and uncompleted ovicells. Moreover, a proportion of the progeny was also brooded in ovicells of females not visible at the moment of allosperm dosage, within and beyond the initial growing edge (Table 11 and 12). The highest proportion of the progeny produced by colonies exposed to two allosperm suspensions was concentrated in ovicells produced within the initial growing edge. These results were independent of which allosperm was dosed first (Table 11 and 12). When allosperm from clone A_1 was dosed first, the proportion of progeny produced by ovicells within the initial perimeter ranged from 0.87 to 0.92 in the short interval and from 0.94 to 1 in the long interval. These proportions were significantly higher than the proportions of progeny produced by females zooids budded beyond the initial growing edge ($t=4.05$; $DF=4$; $P<0.05$, short interval; and $t=6.28$; $DF=4$; $P<0.001$, long interval). Similarly, when allosperm from clone M_1 were dosed first, the proportion of progeny produced by ovicells within the initial perimeter ranged from 0.89 to 1 in the short interval and from 0.91 to 0.97 in the long interval. These proportions were significantly higher than the proportion of progeny produced by females zooids budded beyond the initial growing edge ($t= -6.28$; $DF=4$; $P<0.001$, short interval; $t= -23.24$; $DF=4$; $P<0.001$, long interval).

6.3.4.4 Sexual allocation

ANOVA showed that at the beginning of the experiment there was no significant difference in colony size between colonies exposed to allosperm suspensions and those maintained in reproductive isolation ($F=1.03$; $DF=1$; $P>0.05$). Similarly, ANOVA showed no significant difference in size after 4 wk between colonies exposed to

allosperm and the control colonies (F=1.56; DF=1; P>0.05) (Table 13). Male and female zooids were present in all the colonies at the time allosperm dosage took place. Values of sex ratio and sexual investment before allosperm dosage and 4 wk later are summarised in Table 13. At the beginning of the experiment, ANOVA showed no significant difference in sex ratio and sexual investment between colonies exposed to allosperm and the controls (F=3.02; DF=1; P>0.05, for sex ratio; F=0.76; DF=1; P>0.05). Although during the experimental period sexual zooids were produced in all the experimental colonies, after 4 wk values of sex ratio and sexual investment were significantly higher in colonies exposed to allosperm than in control colonies (F=48.82; DF=1;P<0.0001, for sex ratio; F=11.61; DF=1; P<0.001, for sexual investment).

6.3.4.5 Fingerprint analysis and sperm precedence

The proportion of embryos becoming a 3 wk old colony was always over 0.80, and the proportion of these colonies from which zooids were removed for fingerprint analyses ranged from 0.75 to 0.98 (Tables 9 and 10). Fingerprint analysis of the progeny showed low values for P_2, i.e. most of the progeny was fathered by the first allosperm to which the receptor colonies were exposed. With the exception of two cases, it was found that when a short interval between double allosperm was used and M_1 was the first donor, first-allosperm precedence was always found (Tables 9 and 10). In the first exception, the progeny produced by colony H_1 (Table 10) were fathered in similar proportion by both mates. In the second exception, a high value of P_2 suggesting second-male precedence was found when the progeny of J_1 were analyzed (Table 10).

When allosperm from clone A_1 was dosed first, the proportion of progeny fathered by this clone ranged from 0.78 to 0.94 and from 0.80 to 0.88 in the short and long interval between matings respectively (Table 10). The proportion of progeny fathered by the first mate (P_1) was significantly higher than the proportion of the progeny fathered by the second mate (P_2). This result was found in both the short and long interval between the first and second mating (t=6.18; DF= 4; P<0.01, short interval; t=21.51; DF=4; P<0.0001, long interval).

When allosperm from M_1 was dosed first (excluding the anomalous progeny from J_1),

progeny fathered by the first allosperm ranged from 0.58 to 0.86 and from 0.74 to 0.96 in the short and long interval between the matings respectively (Table 9). No significant differences in proportion of the progeny fathered by allosperm from clone M_1 or clone A_1 with a short interval between matings were found (t= -1.36; DF= 4; P>0.05). However, excluding from the analysis the anomalous trends found in progeny form J_1, these differences became significant (t= -3.55; DF= 3; P<0.05). Analysis of data from the long interval between matings showed that the proportion of progeny fathered by the first mate (P_1) was significantly higher than the proportion fathered by the second mate (P_2) (t= -6.42; DF= 4; P<0.05).

Fingerprint analysis from zooids of the first and last 20 settled colonies are summarized in Tables 14 and 15. Analysis of the first settled colonies showed that when a short interval between the two allosperm dosages was used, the proportions of progeny fathered by each allosperm donor were not significantly different (t=0.65; DF= 4; P>0.05, when A_1 was the first donor; and t=0.75; DF= 4; P>0.05, when M_1 was the first donor). However, when a long interval between matings was used, significantly higher proportion of the progeny was fathered by the first allosperm donor (t=21.5; DF= 4; P<0.0001, when A_1 was the first donor; and t= -21; DF= 4; P<0.0001, when M_1 was the first donor).

Analysis of the last settled colonies showed that when a short interval between the two matings was used, a significantly higher proportion of the progeny was fathered by the first allosperm when clone A_1 was used as first donor (t= 4.40; DF= 4; P<0.05). Similarly, when a long interval between matings was used a significantly higher proportion of the progeny was fathered by the first allosperm donor (t=7.21; DF=4; P<0.01). However, when clone M_1 was used as first donor as a consequence of the anomalous progeny from J_1, the proportion of progeny fathered by the first allosperm was not significantly higher than for the second donor (t= -1.70; DF= 4; P>0.05). When progeny produced by clone J_1 were not considered in the analysis, the proportion of progeny fathered by the first allosperm was significantly higher than the proportion fathered by the second donor (t= -4.75; DF= 3; P<0.05).

6.4 Discussion

Presence of progeny in all the colonies exposed to single allosperm suspensions is in agreement with results of the preliminary experiments where sexual compatibility between the experimental clones was assessed. This confirms that receptor colonies exposed sequentially to two different allosperm suspensions were indeed exposed to compatible and functional allosperm. On the other hand, absence of visible oocytes and production of progeny in the control colonies suggests that self-fertilization is rare. Again, this result is in agreement with findings of other experiments in this dissertation. Appearance of the first oocytes during the first week and the latest ones within the fifth and sixth week after exposure to allosperm suspensions are in agreement with previous results on brooding time (see Chapter 3).

Significant increase in sex ratio and sexual allocation in colonies exposed to allosperm suspensions are in agreement with results where the effect of exposure to allosperm on sexual investment was studied (see Chapter 7). These results suggest that in colonies of C. hyalina sexual allocation is positively modulated by the presence of allosperm. These increments in sexual investment involved the production of more female zooids than males, suggesting that female budding is triggered by the onset of sexual reproduction. Moreover, production of male zooids was primarily in frontal budding, in agreement with previous findings showing that basal males are mainly produced under stress conditions in the laboratory (see Chapter 7) and are not common in colonies growing in the field (personal observation).

A negative effect of sexual investment on colony size has been found only under continuous exposure to allosperm (see Chapter 7). In colonies exposed for only one day to allosperm suspensions no negative effects of the onset of sexual reproduction on colony size were found (see Chapter 7). This is in agreement with results of the present study, where no differences on colony size were found in control colonies and colonies exposed to allosperm suspensions.

Appearance of progeny in ovicells produced beyond the initial growing edge of the colonies confirms presence of sperm storage and intracolonial sperm movement. These

results are in agreement with previous findings (see Chapter 3).

Fingerprint analyses corroborate the absence of progeny in the control colonies and strongly suggest prevalence of outcrossing. In 0.16% of the reliable samples (N=1248), i.e. where the score was not ambiguous or attributed to sperm contamination, the presence of only the maternal alleles at the scored locus without a third paternal allele in the same gel track suggests that these progeny was the product of self-fertilization. Analysis of progeny exposed sequentially to two allosperm suspensions showed that the progeny were fathered by both sources of allosperm, suggesting allosperm mixing. However, on average more progeny were fathered by the first allosperm to which the receptor colonies were exposed. Similar results were found when allosperm dosages were 2 h or 48 h apart. This shows that the first allosperm in contact with the receptor colonies are more likely to fertilze eggs than the allosperm dosed later, suggesting a first-allosperm precedence. Since a high proportion of the embryos produced by the receptor colonies was able to produce a 3 weeks old colony and a high proportion of these colonies was included in the samples for fingerprinting, the results of this study cannot be attributable to differential mortality or biased sampling method. Negative effects of paternal relatedness on progeny mortality have been shown in several animal taxa. This negative effect of inbreeding is a potential confounding effect in studies of sperm competition and sperm choice (Olson et al., 1999). However, although relatedness among the experimental parental colonies was not assessed, high values of fertilization success suggest that the experimental design was not biased as consequence of inbreeding depression. Results on sperm longevity in C. hyalina show that fertilization success declines rapidly after 1 h (see Chapter 3). However, in the present study sperm from the first and second donors were not collected at the same time. Consequently, sperm from the second donor were not at an unnatural disadvantage and first precendece cannot be attributed to sperm ageing or differential sperm fertilization capacity. Thus, the interpretation of the results should be considered only in terms of sperm precedence.

Sperm precedence in the long interval treatment can be attributed to an experimental artefact due to allosperm from the first dosage using up the initial pool of oocytes and the second dosage using newly produced oocytes. This first-in / first-out bias in sperm

precedence favouring early sperm could happen if the first sperm are stored closer to the anatomical place were fertilization takes place. Moreover, existence of physiological or biological sperm plugs triggered by early sperm could also acting against latest sperm. However, since acquired sperm are stored up to 5 wk, female cryptic choice could take place. Female interests are important in determining paternity (Birkhead and Parker, 1997), therefore selection by females favouring stored allosperm cannot be dismissed. The present work cannot rule out such mechanisms of female choice, but there is no evidence in favour of their existence.

Since no differences in colony size were detected either at the beginning or at the end of the experiment, roughly similar numbers of progeny produced by single and double-mated colonies suggest a trade-off between colony size and sexual reproduction. However, under continuous allosperm exposure colonies of similar size could reach higher values of sexual allocation and higher production of progeny (personal observations). This suggests that the lack of extra progeny produced by the double-mated colonies was a consequence of limitations in the capacity of sperm storage. Although double-mated colonies were exposed twice to allosperm suspensions, colonies in both mating regimes were able to store similar amounts of sperm. The possibility of limitation in number of oocytes to be fertilized cannot be discounted. The only case where more progeny was fathered by the second allosperm was found with a short interval between the first and second mating, with allosperm from clone A_1 being used as the second mating (Table 10). However, P_1 precedence was found when allosperm from clone M_1 was used for the second mating (Table 9), and in all the other combinations.

Since brooding time in C. hyalina is relatively constant (see Chapter 3), first settled larvae probably were the product of the first sperm used to fertilize oocytes. Roughly equal values of P_1 and P_2 found for the first settled progeny from the short interval treatment suggests absence of female preference for first allosperm via sperm selection, i.e. sperm mixing occurred. Similarly, equal values of P_1 and P_2 were found when receptor colonies of C. hyalina were exposed simultaneously to two sexually compatible allosperm suspensions (see Chapter 5). This suggests that in simultaneous (Chapter 5) and close sequential matings (present Chapter) females may not be capable of sperm

selection. However, P_2 values of the progeny originated by the first settled larvae are not comparable with P_2 values computed from the full set of progeny produced by colonies used in the sperm competition experiments (Chapter 5). Therefore, it seems more reasonable to base conclusions on information from the latest settled larvae because they should be the product of fertilization by sperm stored in the receptor colonies. Analysis of the latest settled progeny suggests that sperm precedence increases from values expected under random mixing to levels indistinguishable from those expected under first male precedence. A similar trend with low values of P_2 was found when the full set of progeny (i.e. early, late and the progeny settled in between) was used, suggesting first male precedence. The above discrepancy in P_2 values suggests that female mechanism of post copulatory (i.e. cryptic) choice for allosperm, forming the basis of mate precedence. might depend on processes occurring during sperm storage more than on the interval between matings.

Results of the present study are not in agreement with studies on sperm precedence in beetles, which have shown that values of P_2 decreased when the interval between copulation with two consecutive males increased from 1 to 6 h (Radwan, 1997). Different patterns of precedence may be adaptive under different circumstances and a general adaptive rule cannot be established. This is illustrated by a range of P_1 precedence to P_2 precedence passing through mixing described in the literature. High values of P_2 or low values of P_1, i.e. high proportion of the progeny fathered by the second mate have been described in several taxa including insects (e.g. Parker and Smith, 1975; Hooper and Siva Jothy, 1996; Carpenter et al., 1997; Cook et al., 1997; Lewis and Jutkiewicz, 1998; Sawada, 1998), birds (e.g. Birkhead and Møller, 1992; Birkhead, 1995), and crustaceans (e.g. Sevigny and Sainte-Marie, 1996). On the other hand, low values of P_2 or high values of P_1 are described in chelicerate arthropods (Austad, 1984) and mammals (Lacey et al., 1997). Finally, mixing or equal values of P_2 an P_1 are described in some insects (e.g. Parker et al., 1990). Colonies of C. hyalina in natural populations should be exposed to a mixture of allosperm suspensions. However, problems associated with the costs of inbreeding by mating with close relatives (see Chapter 7), and prevalence of outcrossing (this Chapter and the others) suggest that natural selection should favour female selection after sperm capture. Moreover, due to the negative effects of sperm dilution on fertilization success (see Appendix 2) and to

the presence of approximately 2 h fertile sperm longevity (see Chapter 2), it seems pertinent to interpret the present first-male precedence results as an adaptive strategy to maximize sperm utilization. Results of the present study strongly suggest that in C. hyalina the first male to mate (i.e. first allosperm) gains advantage in sperm precedence regardless of the temporal interval between the matings and the order of matings. This pattern could be a primary mechanism by which colonies gain high levels of fertilization success, using allosperm in the order captured. This strategy may be adaptive in the marine environment where fertilization by aquasperm is hindered by sperm dilution. The role of female selection seems to be to prevent fertilization by self and closely related sperm and to facilitate matings with unrelated colonies as soon as their sperm are available for utilization.

Table 1: Trial experiment. Cellsporella hyalina clone compatibility and fertilization success in receptor colonies: exposed to allosperm suspensions from clone A_1 (A); exposed to allosperm from clone M_1 (B); maintained in reproductive isolation from the beginning (C); and in colonies exposed to selfsperm at the beginning of the experiment which remained exposed to selfsperm (D). Note that all the colonies remained exposed to selfsperm.

	Receptor clones					
(A)	E_1	F_1	H_1	J_1	A_2	QD_2
Oocytes	34	55	23	67	36	44
Embryos	38	59	26	72	43	48
Ancestrulae	36	57	25	66	40	44
Fertilization success	0.95	0.97	0.96	0.92	0.93	0.92
(B)	E_1	F_1	H_1	J_1	A_2	QD_2
Oocytes	48	35	63	47	66	74
Embryos	54	39	66	52	73	78
Ancestrulae	52	36	65	46	66	74
Fertilization success	0.96	0.92	0.99	0.89	0.90	0.95
(C)	E_1	F_1	H_1	J_1	A_2	QD_2
Oocytes	0	0	0	0	0	0
Embryos	0	0	0	0	0	0
Ancestrulae	0	0	0	0	0	0
Fertilization success	0	0	0	0	0	0
(D)	E_1	F_1	H_1	J_1	A_2	QD_2
Vitellogenic oocytes	0	0	0	0	0	0
Embryos	0	0	0	0	0	0
Ancestrulae	0	0	0	0	0	0
Fertilization success	0	0	0	0	0	0

Note that in absence of self-fertilization values of fertilization success (FS) are considered as fertilization due to the allosperm suspensions. Values of FS were computed as the number embryos that developed to 3 wk old colonies.

Table 2: Experiment 1, Time of appearance of the first and last ovulated coelomic oocytes in colonies of Celleporella hyalina exposed to allosperm suspension from clone A_1 and M_1. Note that the values are days between the allosperm dosage and the appearance of the last vitellogenic oocyte. In absence of self-fertilization this period is considered as the maximum sperm storage. Last column corresponds to the mean values and standard deviation

	Receptor clones				
	E_1	J_1	A_2	QD_2	Mean (SD)
Allosperm from A_1					
First oocyte	6	5	5	4	5.00 (0.82)
Last oocyte	38	33	31	35	34.25 (2.99)
Allosperm from M_1					
First oocyte	5	4	6	7	5.50 (1.29)
Last oocyte	31	37	33	26	31.75 (4.57)
First A_1 and then M_1					
First oocyte	6	7	8	4	6.25 (1.71)
Last oocyte	33	35	28	30	31.50 (3.11)
First M_1 and then A_1					
First oocyte	4	3	7	6	5.00 (0.83)
Last oocyte	36	34	30	28	32.00 (3.65)

Table 3: Experiment 1, Number of progeny produced by receptor clones of C. hyalina when: allosperm from the clone A_1 were dosed first and allosperm from the clone M_1 second (A); when allosperm from the clone M_1 were dosed first and allosperm from the clone A_1 second (B); and when colonies were exposed experimentally to selfsperm at the beginning of the experiment and then remained exposed to selfsperm produced by themselves during the experiment (C). Ancestrulae are total numbers (and numbers of ancestrulae used for fingerprinting).

	Receptor clones			
(A)	E_1	J_1	A_2	QD_2
Oocytes	0	34	45	67
Embryos	0	36	54	72
Ancestrulae	0	2 (2)	12 (9)	54 (50)
FS	0	0.06	0.22	0.75
(B)	E_1	J_1	A_2	QD_2
Oocytes	0	65	40	63
Embryos	0	69	43	70
Ancestrulae	0	63 (50)	6 (6)	53 (50)
FS	0	0.91	0.14	0.94
(C)	E_1	J_1	A_2	QD_2
Oocytes	0	0	0	0
Embryos	0	0	0	0
Ancestrulae	0	0	0	0
FS	0	0	0	0

Note that in absence of self-fertilization values of fertilization success are considered as fertilization due to the allosperm suspensions. Values of FS were computed as the number embryos that developed to 3 wk old colonies.

Table 4: Experiment 1. Position of the embryos in the colonies when: allosperm from the clone A_1 were dosed first and allosperm from the clone M_1 second (A) and when allosperm from the clone M_1 were dosed first and allosperm from the clone A_1 second (B). Position of the embryos in the receptor colonies are: embryos brooded within the colonial growing edge at the moment that allosperm dosage occurred (within) or embryos brooded beyond the external growing edge (beyond).

	Receptor clones			
(A)	E_1	J_1	A_2	QD_2
Within	0	30 (0.83)	44 (0.82)	64 (0.89)
complete ovicells	0	7	17	30
incipient ovicells	0	17	23	26
no visible females	0	6	4	8
Bevond	0	6 (0.17)	10 (0.18)	8 (0.11)
(B)	E1	J_1	A_2	QD_2
Within	0	58 (0.84)	38 (0.88)	64 (0.91)
complete ovicells	0	14	11	21
incipient ovicells	0	40	20	33
no visible females	0	4	7	10
Bevond	0	11 (0.16)	5 (0.12)	6 (0.09)

Note: values in parentheses are proportion of embryos in the corresponding location in the receptor colonies.

Table 5: Experiment 2, Time of appearance of the first and last ovulated coelomic oocytes in colonies of Celleporella hyalina exposed to allosperm suspension from clone A_1 and M_1. Note that the values are days between the allosperm dosage and the appearance of the last vitellogenic oocyte. In absence of self-fertilization this period is considered as the maximum sperm storage. Last column correspond to mean values and standard deviation

	Receptor clones					
	F_1	H_1	J_1	A_2	QD_2	Mean (SD)
Allosperm from A1						
First oocyte	6	4	5	3	5	4.6 (1.1)
Last oocyte	35	25	38	29	31	31.6 (5.1)
Allosperm from M1						
First oocyte	4	6	6	5	4	5.0 (1.0)
Last oocyte	33	30	35	32	28	31.6 (2.7)
First A_1 and then to M_1						
First oocyte	6	7	4	3	3	4.6 (1.8)
Last oocyte	30	36	39	24	32	32.2 (5.8)
First M_1 and then to A_1						
First oocyte	5	5	4	2	4	4.0 (1.2)
Last oocyte	39	34	34	35	31	34.6 (2.9)

Table 6: Experiment 2, Summary of number of embryos, 3wk old ancestrulae, fertilization success (FS); number of progeny scored or analysed by microsatellite fingerprint (scored ancestrulae / total ancestrulae) and number (proportion) of the progeny fathered by each allosperm donor when receptor colonies were: (A) exposed to allosperm from the clone A_1 first and secondly to allosperm from the clone M_1 and (B) exposed to allosperm from the clone M_1 first and secondly to allosperm from clone A_1. Note that fertilization success is computed as the number of developing embryos becoming viable ancestrulae.

	Receptor clones				
(A)	F_1	H_1	J_1	A_2	QD_2
Total embryos	58	49	51	34	36
3 wk old ancestrulae	48	48	48	34	34
FS	0.89	0.99	0.94	1.00	0.94
Scored samples	42 (0.89)	34 (0.71)	43 (0.90)	30 (0.88)	26 (0.77)
A_1 as father (P1)	3 (0.07)	2 (0.06)	0 (0)	6 (0.20)	2 (0.08)
M_1 as father (P_2)	39 (**0.93**)	32 (**0.94**)	43 (**1.00**)	24 (**0.80**)	24 (**0.92**)
Selfing	0	0	0	0	0
(B)	F_1	H_1	J_1	A_2	QD_2
Total embryos	55	48	63	35	35
3 wk old ancestrulae	48	48	45	33	30
FS	0.87	1.00	0.71	0.94	0.86
Scored samples	47 (0.98)	35 (0.73)	40 (0.89)	30 (0.86)	25 (0.83)
A_1 as father (P_2)	4 (0.08)	1 (0.03)	3 (0.08)	1(0.03)	1 (0.04)
M_1 as father (P1)	43 (**0.92**)	34 (**0.97**)	37 (**0.92**)	29 (**0.97**)	24 (**0.96**)
Selfing	0	0	0	0	0

Table 7: Experiment 2, Position of the embryos when the receptor colonies were: exposed to allosperm from the clone A_1 first and secondly to allosperm from the clone M_1 (A) and exposed to allosperm from the clone M_1 first and secondly to allosperm from clone A_1 (B). Position of the embryos in the receptor colonies are: embryos brooded within the colonial growing edge at the moment that allosperm dosage occurred (within) or embryos brooded beyond the external growing edge (beyond). Moreover, numbers are show as number of embryos in the corresponding location (i.e. complete ovicells, incipient ovicells and no visible ovicells) and as the proportion of the total number of embryos. Note that embryos produced beyond the original growing edge correspond to those considered as result of intracolonial sperm movement in absence of self- fertilization.

	Receptor clones				
(A)	F_1	H_1	J_1	A_2	QD_2
Within	50 (0.86)	47 (0.96)	44 (0.93)	28 (0.84)	31 (0.86)
complete ovicells	10 (0.20)	9 (0.19)	17 (0.38)	7 (0.25)	10 (0.32)
incipient ovicells	23 (0.46)	24 (0.51)	14 (0.32)	12 (0.43)	7 (0.23)
no visible females	17 (0.34)	14 (0.30)	13 (0.30)	9 (0.32)	14 (0.45)
Bevond	8 (0.14)	2 (0.04)	7 (0.07)	6 (0.16)	5 (0.14)
(B)	F_1	H_1	J_1	A_2	QD_2
Within	51 (0.93)	40 (0.89)	60 (0.82)	26 (0.87)	26 (0.93)
complete ovicells	17 (0.33)	6 (0.15)	13 (0.22)	2 (0.08)	4 (0.15)
incipient ovicells	12 (0.24)	14 (0.35)	20 (0.33)	10 (0.39)	16 (0.62)
no visible females	22 (0.43)	20 (0.50)	27 (0.45)	14 (0.54)	6 (0.23)
Bevond	4 (0.07)	5 (0.11)	13 (0.18)	4 (0.13)	2 (0.07)

Table 8: Experiment 3, Time of appearance of the first and last ovulated coelomic oocytes in colonies of Celleporella hyalina exposed to two allosperm suspensions. Data are results when receptor colonies were (A) exposed first to allosperm suspension from clone A_1 and secondly to allosperm suspensions from clone M_1 and (B) when receptor colonies were exposed first to allosperm suspension from clone M_1 and to allosperm suspensions from clone A_1 (B). Note that the values are days between the allosperm dosage and the appearance of the last vitellogenic oocyte. In absence of self-fertilization this period is considered as the maximum sperm storage. Last column correspond to mean values and standard deviation

	Receptor clones					
(A)	F_1	H_1	J_1	A_2	QD_2	Mean (SD)
A_1 (short and long interval)						
First oocyte	4	4	3	6	5	4.4 (1.1)
Last oocyte	38	34	32	34	27	33.0 (4.0)
M_1 (short interval)						
First oocyte	3	5	6	5	4	4.6 (1.1)
Last oocyte	29	32	37	31	35	32.8 (3.19)
M_1 (long interval)						
First oocyte	3	2	2	4	5	3.2 (1.3)
Last oocyte	32	36	28	27	34	31.4 (3.85)
A_1 and then M_1 (short interval)						
First oocyte	4	4	5	3	3	3.8 (0.8)
Last oocyte	36	31	35	29	35	33.2 (3.0)
A_1 and then M_1 (long interval)						
First oocyte	2	4	3	6	3	3.6 (1.5)
Last oocyte	28	38	34	36	40	35.2 (4.6)
(B)	F_1	H_1	J_1	A_2	QD_2	Mean (SD)
M_1 (short and long interval)						
First oocyte	5	4	7	3	5	4.8 (1.5)
Last oocyte	34	31	47	30	29	34.2 (7.4)
A_1 (short interval)						
First oocyte	6	3	5	5	4	4.6 (1.1)
Last oocyte	30	30	32	36	27	31.0 (3.3)
A_1 (long interval)						
First oocyte	4	5	2	4	5	4.0 (1.2)
Last oocyte	32	38	26	41	33	34.0 (5.8)
M_1 and then A_1 (short interval)						
First oocyte	4	3	6	5	6	4.8 (1.3)
Last oocyte	32	36	30	29	36	32.6 (3.3)
M_1 and then A_1 (long interval)						
First oocyte	6	3	4	5	3	4.2 (1.3)
Last oocyte	36	27	39	32	34	33.6 (4.5)

Table 9: Experiment 2, Summary of number of embryos, 3wk old ancestrulae, fertilization success (FS); number of progeny scored or analysed by microsatellite fingerprint (scored ancestrulae/ total ancestrulae) and number (proportion) of the progeny fathered by each allosperm donor when receptor colonies were: (A) exposed to allosperm from the clone A_1 first and secondly to allosperm from the clone M_1 and (B) exposed to allosperm from the clone M_1 first and secondly to allosperm from clone A_1. Note that fertilization success is computed as the number of developing embryos becoming viable ancestrulae. Top part of the table are data for the short interval of 2h in between the two dosages and the bottom part of the table are data for the long interval of 48h in between the two allosperm dosages.

	Receptor clones				
Short interval (2 h)	F_1	H_1	J_1	A_2	QD_2
Total embryos	86	58	61	67	124
3 wk old ancestrulae	77	56	53	60	120
FS	0.90	0.97	0.87	0.90	0.97
Scored samples	60 (0.78)	50 (0.89)	50 (0.94)	50 (0.83)	100 (0.83)
A_1 as father (P_1)	39 (0.68)	39 (0.78)	37 (0.74)	40 (0.80)	61 (0.62)
M_1 as father (P_2)	18 (0.32)	11 (0.22)	13 (0.26)	9 (0.18)	37 (0.38)
Selfing	0	0	0	0	0
Long interval (48 h)	F_1	H_1	J_1	A_2	QD_2
Total embryos	73	53	65	62	60
3 wk old ancestrulae	68	51	64	56	55
FS	0.93	0.96	0.99	0.90	0.92
Scored samples	50 (0.74)	50 (0.98)	50 (0.78)	50 (0.89)	50 (0.91)
A_1 as father (P_1)	40 (0.80)	42 (0.84)	42 (0.85)	42 (0.84)	44 (0.88)
M_1 as father (P_2)	10 (0.20)	8 (0.16)	7 (0.15)	8 (0.16)	6 (0.12)
Selfing	0	0	0	0	0

Table 10: Experiment 3, Summary of number of embryos, 3wk old ancestrulae, fertilization success (FS); number of progeny scored or analysed by microsatellite fingerprint (scored ancestrulae/ total ancestrulae) and number (proportion) of the progeny fathered by each allosperm donor when receptor colonies were: (A) exposed to allosperm from the clone A_1 first and secondly to allosperm from the clone M_1 and (B) exposed to allosperm from the clone M_1 first and secondly to allosperm from clone A_1. Note that fertilization success is computed as the number of developing embryos becoming viable ancestrulae. Top part of the table are data for the short interval of 2h in between the two dosages and the bottom part of the table are data for the long interval of 48h in between the two allosperm dosages.

	Receptor clones				
Short interval (2h)					
Total embryos	F_1	H_1	J_1	A_2	QD_2
3 wk old ancestrulae	64	79	62	119	82
FS	60	75	54	107	66
Scored samples	0.94	0.95	0.87	0.90	0.80
A_1 as father (P_2)	60 (1.00)	70 (0.93)	50 (0.93)	100 (0.93)	60 (0.91)
M_1 as father (P_1)	14 (0.24)	29 (0.42)	36 (0.72)	29 (0.30)	8 (0.14)
Selfing	34 (0.76)	40 (0.58)	14 (0.28)	67 (0.69)	49 (0.86)
Long interval (48h)	0	0	0	1 (0.01)	0
Total embryos	F_1	H_1	J_1	A_2	QD_2
3 wk old ancestrulae	62	78	73	84	76
FS	61	74	62	67	62
Scored samples	0.98	0.95	0.93	0.80	0.82
A_1 as father (P_2)	50 (0.82)	60 (0.81)	50 (0.81)	50 (0.75)	60 (0.97)
M_1 as father (P_1)	13 (0.26)	10 (0.17)	2 (0.04)	11 (0.23)	6 (0.10)
Selfing	37 (0.74)	48 (0.83)	48 (0.96)	37 (0.77)	49 (0.88)
	0	0	0	0	1 (0.02)

Table 11: Experiment 3, Position of the embryos in receptor colonies. Receptor colonies were exposed first to allosperm suspension from clone A_1 and secondly to allosperm suspensions from clone M_1. A short interval of 2h (A) and a long interval of 48h (B) were used in between the two allosperm dosages. Within and beyond are number of embryos in each position (and proportion of the total number of embryos) of the of progeny produced by the receptor colonies. Position of the embryos within the original growing edge are show as number of embryos in the corresponding location (i.e. complete ovicells, incipient ovicells and no visible ovicells) and as the proportion of the total number of embryos. Note that embryos produced beyond the original growing edge correspond to those considered as result of intracolonial sperm movement in absence of self-fertilization.

	Receptor clones				
(A)	F_1	H_1	J_1	A_2	QD_2
Within	75 (0.87)	50 (0.91)	55 (0.90)	61 (0.90)	114 (0.92)
Complete ovicells	21 (0.28)	12 (0.24)	12 (0.22)	15 (0.25)	39 (0.34)
Incipient ovicells	26 (0.35)	18 (0.36)	10 (0.18)	6 (0.10)	34 (0.30)
no visible female	28 (0.37)	20 (0.40)	33 (0.60)	40 (0.65)	41 (0.36)
Beyond	11 (0.13)	8 (0.09)	6 (0.10)	6 (0.10)	10 (0.08)
(B)	F_1	H_1	J_1	A_2	QD_2
Within	69 (0.95)	52 (0.98)	61 (0.94)	62 (1.00)	54 (0.90)
Complete ovicells	15 (0.22)	25 (0.48)	9 (0.15)	18 (0.29)	12 (0.22)
Incipient ovicells	14 (0.20)	21 (0.40)	19 (0.31)	4 (0.06)	21(0.38)
Females no visible	40 (0.58)	6 (0.12)	33 (0.54)	40 (0.65)	21 (0.38)
Beyond	4 (0.05)	1 (0.02)	4 (0.06)	0 (0.00)	6 (0.10)

Table 12: Experiment 3, Celleporella hyalina position of the embryos in receptor colonies. Receptor colonies were exposed first to allosperm suspension from clone M_1 and secondly to allosperm from clone A_1. A short interval of 2h (A) and a long interval of 48h (B) were used in between the two allosperm dosages. Within and beyond are number of embryos in each position (and proportion of the total number of embryos) of the of progeny produced by the receptor colonies. Position of the embryos within the original growing edge is show as: number of embryos in the corresponding location (i.e. complete ovicells, incipient ovicells and no visible ovicells) and as the proportion of the total number of embryos. Note that embryos produced beyond the original growing edge correspond to those considered as result of intracolonial sperm movement in absence of self-fertilization.

	Receptor clones				
(A)	F_1	H_1	J_1	A_2	QD_2
Within	57 (0.89)	75 (0.95)	60 (0.97)	91 (0.76)	82 (1.00)
complete ovicells	10 (0.18)	30 (0.40)	14 (0.23)	35 (0.39)	34 (0.41)
incipient ovicells	35 (0.61)	13 (0.17)	21 (0.35)	33 (0.36)	35 (0.43)
no visible females	12 (0.21)	32 (0.43)	25 (0.42)	23 (0.25)	13 (0.16)
Beyond	7 (0.11)	4 (0.05)	2 (0.03)	28 (0.24)	0 (0.00)
(B)	F_1	H_1	J_1	A_2	QD_2
Within	60 (0.97)	71 (0.91)	68 (0.93)	77 (0.92)	71 (0.93)
complete ovicells	15 (0.25)	12 (0.17)	19 (0.28)	33 (0.43)	28 (0.39)
incipience ovicells	25 (0.42)	36 (0.51)	26 (0.38)	24 (0.31)	23 (0.32)
females no visible	20 (0.33)	23 (0.32)	23 (0.34)	20 (0.26)	20 (0.28)
Beyond	2 (0.03)	7 (0.09)	5 (0.07)	7 (0.08)	5 (0.07)

Table 13: Experiment 3, Mean values of colony size (CS), sex ratio (SR) and sexual allocation (SA) in colonies of Celleporella hyalina exposed to allosperm suspensions (i.e. allosperm from clone A_1 and clone M_1) and in control colonies maintained in reproductive isolation. CS are data in mm^2, SR were computed dividing number of female zooids by number of male zooids in the colonies and, SA were computed dividing the number of sexual zooids (i.e. males and females) by the total number of basal autozooids in the colonies. Values of CS, SR and SA were respectively log, log+1 and arcsin transformed before the ANOVA were conducted. Note * = P<0.05.

	One day before allosperm dosage			Four weeks after allosperm exposure		
	allosperm	Control	P	Allosperm	control	P
CS	104.37 (12.66)	110.37 (20.97)	NS	217 (19.79)	208.90 (19.27)	NS
SR	0.96 (0.15)	0.87 (0.12)	*	1.69 (0.37)	0.96 (0.12)	*
SA	0.13 (0.03)	0.12 (0.03)	*	0.17 (0.03)	0.12 (0.01)	*

Table 14: Experiment 3. Number and proportion of progeny produced by each allosperm donor in relation to their order of mating. Receptor colonies were exposed first to allosperm suspensions from clone A_1 and secondly to allosperm suspensions from clone M_1 with a short interval of 2h (A) or a long interval of 48h between the two dosages (B).

(A)	First 20 settled ancestrulae		Last 20 settled ancestrulae	
R.C	A_1	M_1	A_1	M_1
F_1	9 (0.45)	11 (0.55)	16 (0.80)	4 (0.20)
H_1	13 (0.65)	7 (0.35)	17 (0.85)	3 (0.15)
J_1	12 (0.60)	8 (0.40)	16 (0.80)	4 (0.20)
A_2	11 (0.55)	9 (0.45)	20 (1.00)	0 (0.00)
QD_2	8 (0.40)	12 (0.60)	15 (0.75)	5 (0.25)
mean proportion (SD)	P_1	P_2	P_1	P_2
	0.54 (0.09)	0.46 (0.10)	0.84 (0.10)	0.16 (0.10)
(B)	First 20 settled ancestrulae		Last 20 settled ancestrulae	
R.C	A_1	M_1	A_1	M_1
F_1	18 (0.90)	2 (0.10)	18 (0.90)	2 (0.10)
H_1	20 (1.00)	0 (0.00)	15 (0.75)	5 (0.25)
J_1	18 (0.90)	2 (0.10)	14 (0.70)	6 (0.30)
A_2	19 (0.95)	1 (0.05)	16 (0.80)	4 (0.20)
QD_2	18 (0.90)	2 (0.10)	17 (0.85)	3 (0.15)
mean proportion (SD)	P_1	P_2	P_1	P_2
	0.93 (0.04)	0.07 (0.04)	0.80 (0.08)	0.20 (0.08)

Table 15: Experiment 3. Number and proportion of progeny produced by each allosperm donor in relation to their order of mating. Receptor colonies were exposed first to allosperm suspensions from clone M_1 and secondly to allosperm suspensions from clone A_1 with a short interval of 2h (A) or a long interval of 48h between the two dosages (B).

(A)	First 20 settled ancestrulae		Last 20 settled ancestrulae	
R.C	M_1	A_1	M_1	A_1
F_1	12 (0.60)	8 (0.40)	17 (0.85)	3 (0.15)
H_1	10 (0.50)	10 (0.50)	16 (0.80)	4 (0.20)
J_1	9 (0.45)	11 (0.55)	5 (0.25)	15 (0.75)
A_2	9 (0.45)	11 (0.55)	14 (0.70)	6 (0.30)
QD_2	13 (0.65)	7 (0.35)	19 (0.95)	1 (0.05)
mean proportion (SD)	P_1	P_2	P_1	P_2
	0.53 (0.09)	0.47 (0.09)	0.71 (0.27)	0.29 (0.27)
(B)	First 20 settled ancestrulae		Last 20 settled ancestrulae	
R.C	M_1	A_1	M_1	A_1
F_1	18 (0.90)	2 (0.10)	16 (0.80)	4 (0.20)
H_1	18 (0.90)	2 (0.10)	18 (0.80)	4 (0.20)
J_1	20 (1.00)	0 (0.00)	18 (0.90)	2 (0.10)
A_2	18 (0.90)	2 (0.10)	14 (0.70)	6 (0.30)
QD_2	18 (0.90)	2 (0.10)	16 (0.80)	4 (0.20)
Mean proportion (SD)	P_1	P_2	P_1	P_2
	0.92 (0.06)	0.08 (0.06)	0.80 (0.07)	0.20 (0.07)

Chapter 7:
Sexual investment: effects of allosperm exposure and stressors.

7.1 Introduction

In marine invertebrates, gametogenesis is typically controlled by endocrine mechanisms entrained and synchronized by exogenous physical cues of long periodicity (Olive, 1995). Failure to produce fully grown eggs in the absence of another genetic individual has been reported in internally fertilizing invertebrates (Ryland and Bishop, 1990; Cancino et al., 1991). In Chilean C. hyalina investment in female zooids has been reported only in the presence of conspecifics (Cancino et al., 1991). Personal observation of Welsh specimens maintained over a period of two years in reproductive isolation has found an absence of both large vitellogenic oocytes and production of viable progeny. Moreover, low values of sexual investment in comparison with wild, similarly sized colonies, or colonies experimentally exposed to allosperm suspensions, were also found. In ovicellate cheilostome bryozoans, sperm enter the female coelom via the dorsal coelomopore and penetrate primary oocytes in early stages of growth (Temkin, 1996). Fertilization in C. hyalina has been suggested to occur during vitellogenesis or in previtellogenesis by D.J. Hughes (1987). His observations are in agreement with records of sperm heads found in the colemomic cavity of the ooecium and in the coelom of incipient female zooids (Ostrovsky, 1998). Spermatozoids have even been found to have penetrated previtellogenic oocytes (Ostrovsky, 1998). The association of sperm with early ovarian oocytes suggests that allosperm may be the agent responsible for triggering female maturation and the trigger for sexual investment in colonies of C. hyalina. To date, the impact of allosperm on female maturation in C. hyalina is poorly understood.

Animals living in the intertidal zone of marine environments may be exposed to desiccation, osmotic stress, temperature stress, mechanical stress and to biological threats such as competition and predation. Colonies of C. hyalina growing on fronds of Fucus serratus in the Menai Strait are commonly exposed to all these factors. Therefore, in addition to the effects that presence of sexually competent sperm could play in sexual allocation, stresses also may have profound consequences on sexual allocation of colonies living within this habitat.

The present investigation aims to obtain information on the possible waterborne effects of both cellular or smaller sized particles associated with compatible allosperm suspensions on both oocyte growth and sexual allocation in adult virgin colonies of C. hyalina. Furthermore, the effects of exposure to allosperm suspension on the onset of sexual reproduction in sexually immature colonies were studied. Finally, the effects of exposure to allosperm suspensions of different genetic relatedness and both physical and biological stresses on colonial sexual allocation were studied.

7.2 Materials and methods:

7.2.1 Experimental ramets and culture conditions

Experimental clones were stabilised, propagated and reared in reproductive isolation using standard methods (see Appendix 1). All the experiments were conducted in a controlled temperature room set at 15-16°C under similar conditions to those described earlier (see Chapter 2). The experimental colonies were maintained in 300 ml flat-bottomed glass vessels fitted with a plastic lid to ensure sexual isolation throughout the experiments (see Appendix 1). Seawater within the vessel was maintained at a salinity of 34‰ and temperature of 15-16°C. When sperm suspensions were required the light-dark reaction was used and Hoechst 333342 staining done as described in Appendix 2. Ramets were fed daily to achieve a final concentration of 100-200 cell μl^{-1} and transferred every second day to clean vessels containing filtered UV treated sea water (hereafter FSW) that had been aged for 2-3 days. Once a week, ramets and acetate sheets bearing the colonies were cleaned using artist's brushes. In this way, the maximum proportion of active autozooids was sustained, so maximising the capacity of recipient ramets to retain and nourish vitellogenic oocytes and embryos. When larval settlement was required to obtain progeny, settlement units as described in Chapter 2 were used.

Sexual compatibility between donor and receptor clones was assessed in preliminary trials. In these trials, progeny were deemed viable only if they survived for 3 wk after settlement.

7.2.2 Statistical analyses

ANOVA was used to examine the effect of the different treatment on colony size, percentage of active zooids, sex ratio, sexual investment and proportion of sexual zooids. When normality and equality of variance in data set was not achieved, the nonparametric Kruskal-Wallis test was applied. All the statistical analyses were conducted using SPSS 8.0 data analysis program.

7.2.3 Experiment 1: effect of a waterborne factor

The first part of this experiment was carried on between September and November 1997, and the second one between December 1997 and February 1998. Sperm suspensions were obtained using the dark-light reaction and sperm counted using standard methodology (see Appendix 2). From the allosperm suspensions three different treatments were prepared: (a) 800 ml of sperm suspension filtered using a Whatman CN filter (0.45µm pores, cellulose nitrate, Whatman, Maidstone, UK), (b) 800 ml of sperm suspension filtered using a Millipore PVDF filter, (0.45 µm pores, polyvinylidene difluoride, Millipore, Watford, UK) and, (c) as a control, 800 ml of a selfsperm suspension was obtained as above and passed through the filter holder without the filter membrane in place. Proceedings were taken in standard order to obviate ageing effects on sperm performance. The filtering device used in the present study consisted of 1 litre side-arm flasks in which plastic filter funnels had been fitted in the opening through a perforated rubber cork. Vacuum was generated by simultaneously connecting the arm of each flask to the water tap.

In both experiments recipient ramets were acclimatized to experimental conditions by maintaining them individually in 300 ml vessels for at least 1 wk before exposure to the suspensions. In each treatment and in the control, three virgin ramets of the recipient clone were separately exposed to 200 ml of unstirred experimental suspension, with the addition of 0.5 ml of R. reticulata cells for 1 h. The colonies were cleaned with FSW for 5 minutes and assigned to individual experimental vessels (Plate 1). Recipient ramets were chosen to have standard size and percentages of active zooids. The mean number of active zooids per recipient was 242 and 201 zooids in the part 1 and part 2 respectively. Active zooids were recognised by their possession of a complete feeding apparatus.

After being exposed to allosperm suspension, ramets were observed under a Wild stereo microscope every day until the last larva had been released. Counts were made of ovulated and growing coelomic oocytes (hereafter oocytes). Activated eggs or oocytes in vitellogenic phase are easily recognized in the coelom of female zooids in C. hyalina. The numbers of oocytes counted during the experiment were summed for each colony. Drawings of the recipient colonies made on the day before being exposed to the

suspensions were used to locate oocytes in the female coelom, in order to avoid counting errors. Since no developing oocytes were found in any of the experimental ramets during 2-3 mo prior to experimentation, the appearance of the last vitellogenic oocyte was used cautiously to estimate duration of allosperm storage. Colonies were drawn at weekly intervals for 2 mo using a camera lucida connected to the stereo microscope. Drawings were later digitized to obtain colony area and perimeter. At the same time counts of active zooids, recycling zooids, female zooids and male zooids were made. Also recorded were female zooids with a complete brood chamber (hereafter full females), females with an incomplete brood chamber (hereafter incipient females), frontal males and basal males. Values of sex ratio (female zooids/male zooids), sexual allocation (sexual zooids/basal autozooids) were then derived and compared among the treatments. All counts were made using 40 x 10 magnification and a 1200 x 1200 μm graticule placed beneath the experimental substratum.

7.2.4 Experiment 2: effect of physical stressors at the growing edge on sexual allocation

Preliminary observations made during the establishment of the clone bank showed that contact between the growing edge of the colonies and silicone glue retarded colonial growth while promoting the formation of basal males. In the present study, carried out between March and June 1997, these findings were expanded by exploring the effect of two sorts of barriers to colonial growth, i.e. biological and not biological barriers. A biological barrier was considered to be the contact between the growing edges of two colonies while that between the growing edge of a colony and a piece of glass was considered to be not biological. The biological barrier was staged by attaching two colonies growing onto pieces of acetate to a third piece of acetate serving as a common substratum (ca. 45 x 75 mm). The colonies were placed with their growing edges facing each other and initially separated for 4000-5000 μm. Two sorts of contact were created, isocontact (i.e. contact between ramets of the same genotype) and allocontact (i.e. contact between ramets of different genotypes). Seven couples of isocontact and seven of allocontact were studied. Using information generated during the establishment of the clone bank (see Appendix 1), normally growing and slow growing genotypes were selected. Accordingly, 4 normally growing clones (A_1, M_1, E_1 and D_1) and 3 slow growing clones (T_1, F_2 and T_2) were considered. Ramets attached to a piece of acetate without

another colonies were used as controls. In the generation of the allocontact, ramets from clone Q_1 were used. However, control colonies were similar to those used in the isocontacts but allosperm from clone Q_1 were provided every second day in order to emulate the continuous presence of allosperm in the experimental contacts. To generate the not biological barrier, the 7 clones used for biological contact were used (i.e. A_1, M_1, E_1, D_1, T_1, F_2 and T_2). Fragments of acetate bearing ramets from these clones were attached to a third piece of acetate (ca. 45 x 75 mm), separated by 1-2 mm from the growing edge of each colony. A small fragment of glass (ca. 15 x 15 mm and 1 mm thick) was then attached to the third acetate. Ramets of these clones attached to plain acetates without another colony were used as controls.

Once the colonies for biological contact were growing on the common substratum, the first measurement was taken before contact occurred. The numbers of male and female zooids inside a known area close to the growing edge of the colony were counted. These counts were made in 10 random 1600 x 1600 µm squares (ca. 26-28 basal zooids) traced on a transparent acetate placed beneath the colonies. All the squares began at the first row of fully developed basal autozooids inside the growing edge. Fully developed autozooids were considered to be those with a complete and functional feeding apparatus. Measurements were taken from one ramet in each pair in isocontact. Similarly, for allocontacts measurements were not taken from clone Q_1. Measurements were made in the contact area and on the opposite side of the colonies where growth was not constrained. Measurements were repeated once the two colonies were in contact over a length of 0.5 to 0.8 cm. Measurements were made in 10 random 1600 x 1600 µm squares (above) starting from the first row of complete basal autozooids close to the growing edge at the contact zone. In the biological and physical contact, camera lucida drawings of the outline of 20 basal autozooids per clone in the contact area, contact-free area and in equivalent areas in control colonies were processed with an electronic digitizer to assess the basal area of the autozooids.

7.2.5 Experiment 3: effect of not biological disturbance at the colonies on sexual allocation

7.2.5.1 Temperature

Thermal stress was induced by exposing colonies for a short time to temperatures below the normal thermal range to which colonies of the Welsh C. hyalina are exposed in their natural habitat. The water temperature in the Menai Strait varies from 13 to 20°C (Cancino, 1983 Owrid and Ryland, 1993). However, in winter low temperatures can reach values close to 3-5°C (Kawai, personal communication). Therefore, in the present study a temperature of 5 °C was used as stressor. Two virgin ramets of C. hyalina from each of 4 clones were used (A_1, M_1, E_1, and J_1). One ramet was exposed for 2 h to seawater at 4-5°C and the other maintained at the standard culture conditions (15-16 °C). During this time R. reticulata cells were present in the water at a concentration of 100-200 cells.μl^{-1}. After 2 h, all the colonies were assigned to individual 300 ml flat-bottomed glass vessels and maintained under standard conditions for two months before measurements of performance were taken.

Basal autozooids and sexual zooids were counted within a sampling area consisting in 7 squares of 1600 x 1600 µm randomly placed on top of the first basal zooids budded beyond the initial colonial growing edge. Counts were made one month after the stressor was applied. Initial colonial growing edge was taken to be the edge of the colonies before the stressor was applied. Camera lucida drawings were used to visualize the initial edge, to assess the outline of the first basal autozooids and a stereo microscope was used for counts. An electronic digitizer was used later to obtain the basal area of the autozooids.

7.2.5.2 Desiccation

Two ramets of the same clones used in the temperature experiment were used here (A_1, M_1, E_1, and J_1) were used here. One ramet of each clone was desiccated by exposure to air for 10 periods each of 10 min. After each period of desiccation colonies were immersed for 2 min in FSW. Control colonies were continually immersed. Following treatment, colonies were assigned individually to 300 ml flat-bottomed glass vessels and maintained under standard conditions for one month before measurements were taken as in the temperature experiment.

7.2.5.3 Food availability

As in the previous experiment, two ramets of the same clones were used here (A_1, M_1,

E_1, and J_1). The experimental ramets were maintained individually to 300 ml flat-bottomed glass vessels with FSW and no food was supplied for 3 days. The control colonies were maintained in similar containers but food was supplied daily. After the 3 days all the colonies were assigned to clean rearing vessels and maintained under standard conditions (above). The experimental measurements were the same as for the previous stressors, and they were assessed one month after the colonies were assigned the standard culture conditions.

7.2.5.4 Mechanical damage

In this experiment, three ramets of the same clones were used in the previous experiments were used here (A_1, M_1, E_1, and J_1) as for the previous stressors. The mechanical damage included destruction of the distal part of basal autozooids at the growing edge of the colony at 20 points along the growing edge (Treatment 1). At each point destruction took place along 2 mm of the growing edge (ca. 5-6 basal zooids). In the second treatment, total destruction of all the basal autozooids within 20 squares of 1 mm^2 were made in areas located 4-5 mm apart from the growing edge of the colonies (Treatment 2). Finally, control colonies were considered to be colonies where no stress was applied.

For taking measurements, the 1600 x 1600 μm square grid (above) was placed beneath the colonies and the number of basal autozooids and sexual zooids counted within the sampling area. In treatment 1, counts were made at each of the 20 points where the stress was applied, of new basal zooids budded beyond the growing edge. In Treatment 2, all the new zooids budded within the cleared area were counted. Finally, in the control colonies basal zooids were counted in 20 random 1600 x 1600 μm squares placed along the edge of the growing edge of the colonies. In treatments 1 and 2, to assess basal area of the newly budded autozooids, camera lucida drawings of 10 random autozooids were made at 5 of the 20 point where the stress was applied. The basal areas of zooids located in equivalent areas in unstressed colonies were measured and used as controls.

7.2.6 Experiment 4: effect of exposure to sperm suspensions of different genetic relatedness on sexual allocation and fertilization success

Seven virgin colonies from clones AM_1, AE_8, QJ_9 and QD_2 were exposed to sperm suspensions of different degrees of genetic relatedness, including selfsperm, full sib, half sib, maternal and unrelated sperm. One extra ramet per clone was used as a control and not exposed to sperm suspensions. However, these ramets, like the rest, were exposed to autosperm throughout all the experimental period. Sperm suspensions were obtained and sperm counted by using the standard methodology of dark-light reaction and Hoechst 333342 staining (see Appendix 2). Receptor colonies were exposed to the sperm suspensions for 2h in 300 ml flat-bottomed vessels. Care was taken always to use similar, non-limiting sperm concentrations.

The day before exposure to allosperm, colonies were drawn using a camera lucida connected to a stereo microscope. These drawings included the perimeter of the colony and counts of basal male zooids, frontal male zooids, female zooids and recycling autozooids. Total numbers of basal zooids were predicted using regression of number of basal zooids on colony size (see Appendix 3). Colony size was obtained from the camera lucida drawings using an electronic digitizer. Measurements were repeated 2 months later. During this period colonies were maintained individually in flat-bottomed vessels under standard culture conditions (above).

7.2.7 Experiment 5: effect of continuous exposure to allosperm on the onset of sexual reproduction and sexual allocation.

7.2.7.1 From 10 basal autozooids (sexually mature colonies)

From each of 6 clonally propagated virgin clones, 5 ramets were prepared using the method described above. After 3 wk, once the colonies had grown onto the experimental substratum, the founder piece of acetate was carefully removed, so those colonies were growing on a homogenous surface. In order to standardize the initial number of zooids, unwanted zooids were scraped away from each colony. Care was taken to reduce all the colonies to exactly 10 active autozooids, without leaving any early stage, cup-shaped frontal sexual buds or fully developed sexual zooids.

Two different experimental runs were made using the same clones and experimental

protocol. The first experimental run was made between November 1997 and January 1998 with 18 months old cultured clones. The second experimental run was made between December 1998 and January 1999 when the experimental clones were 30 months old. The experimental clones were A_1, D_1, E_1, J_1, M_1 and Q_1. These clones had been maintained under strict reproductive isolation from the moment that the founder colonies were established in May 1996 from a single larva.

One control ramet of each clone was assigned to an individual culture vessel and not exposed to allosperm. The remaining two ramets were exposed for 2 h to a fresh allosperm suspension (Treatment 1 and Treatment 2). The allosperm suspension was made of fresh sperm obtained from clones A_1, M_1 and E_1. After exposure to allosperm, the ramets were rinsed with 0.2 µm FSW then placed in clean experimental vessels under standard culture conditions. After exposure to allosperm one set ramets was maintained under strict reproductive isolation for 70 d (Treatment 1). This treatment allowed us to investigate the effect of a short exposure to allosperm on the onset of male and female function. Moreover, we could investigate whether or not these small colonies, without any visible female zooids, were able to store allosperm. The other set of ramets was exposed daily from the second day to sperm suspension from the clone N_2. This treatment allowed us to investigate the effect of continuous exposure to allosperm on the production of sexual zooids, sexual allocation and growth rate. Moreover, using DNA fingerprinting the progeny was investigated to assess if they were fathered by the allosperm from the allosperm cocktail or by first allosperm offered during the first day of experimentation (see Chapter 6 for results on paternity analyses). Since not visible female zooids were presents in the receptor colonies at the beginning of the experiments, progeny fathered by the allosperm offered during the first experimental day were used as evidence of sperm storage.

In preliminary trials, reproductive compatibility between all the experimental clones and the allosperm donors was found. For more than two years the 6 clones involved in the present study had not produced any larvae. Moreover, trial experiments exposing ramets of each clone to selfsperm solutions had shown that self-fertilization does not occur under strict reproductive isolation.

7.2.7.2 From ancestrulae (sexually immature colonies)

In the present experiment four sets of full sib colonies of C. hyalina were used. These colonies were obtained from manipulated matings made in the laboratory. The full sib colonies were obtained by mating: a) one ramet of clone A_1 with allosperm from clone M_1; b) one ramet of clone A_1 with allosperm from clone E_1; c) one ramet of clone Q_1 with allosperm from clone J_1; and d) one ramet of clone Q_1 with allosperm from clone D_1. Ramets exposed to allosperm were maintained in reproductive isolation until larvae were ready to be released. To induce larval release, colonies were maintained in darkness overnight and then exposed to light. From each maternal colony, 6 larvae were collected with the aid of a pipette and placed individually in settlement units. Plastic multiwell plates filled with 5 ml of FSW were used as settlement units. In the bottom of each well was placed a circular piece of conditioned acetate. Acetate sheets were conditioned by maintaining them in running seawater for more than 2 mo. Once the larvae had settled, the water in each well was changed and microalgal food supplied. After 2 d small sections of 0.4 x 0.4 cm bearing the postmetamorphic ancestrulae were carefully trimmed and glued with cyanocrylate to the centre of individual acetate sheets (7.5 x 4.5 cm). The three most healthy looking full sib colonies produced by each mating were chosen. Each colony was placed in separate flat-bottomed glass vessels closed with perforated plastic cover, similar to those used in the Experiment 1.

After 2 wk, when they were 2 active zooids in size (0.3-0.4 mm^2), the small colonies were assigned to the experimental treatments. One full sib colony of each trio was maintained in reproductive isolation (Control). The remaining full sib colonies were simultaneously exposed for 2 h to an allosperm solution from clone H_1 (Treatment 1 and Treatment 2). The colonies were then cleaned with FSW and one copy of each couple was maintained separately in reproductive isolation for 70 d (Treatment 1). This treatment allowed us to investigate the effect of a short exposure to allosperm on the onset of sexual activity and sperm storage. The remaining full sib colonies were exposed daily to sperm suspensions from clone N_2 (Treatment 2). This treatment allowed us to investigate the effect of a continuous exposure to allosperm on the onset of sexual activity and sexual allocation. Using DNA fingerprinting the paternity of the progeny was investigated.

In experiments from 10 basal zooids and those from ancestrulae, the acetate sheets bearing the experimental colonies were placed individually in flat-bottomed glass vessels

closed with perforated plastic (see Appendix 1). The colonies were fed daily using R. reticulata suspension and the water in the experimental vessels changed every second day with 48 d aged FSW. The colonies were cleaned every week using a soft art bush and the vessels replaced by clean ones. Care was taken to clean once a week the bubbling system and the lids, to remove unwanted settled algae. All the experimental colonies were maintained in 300 m flat-bottomed glass vessels (see Appendix 1) in a constant temperature room as described above.

7.3 Results

7.3.1 Experiment 1: filter experiment

7.3.1.1 Sperm count

In parts 1 and 2 of this experiment, sperm counts showed presence of sperm cells only in the unfiltered allosperm and selfsperm suspensions. No sperm cells were detected in the suspension filtered through Whatman or Millipore filters.

7.3.1.2 Oocytes

In both parts of the experiment, the presence of oocytes in the female zooids was only found in those receptor colonies exposed to unfiltered allosperm suspensions (Fig. 3). The appearance of the first oocytes in the receptor colonies exposed to unfiltered allosperm suspension was by day 3 and 5 in part 1 and 2 respectively. Meanwhile, the last oocytes were observed on day 38 and 55 respectively. No oocytes were found in the receptor colonies exposed to allosperm suspension filtered using Whatman or Millipore filters. No oocytes were found in those receptor colonies exposed to selfsperm suspensions (Fig. 3). Oocytes were observed in full and incipient females zooids present in the receptor colonies at the moment of allosperm exposure, as in female zooids budded later. These female zooids were budded within the initial margins of the colonies, as in females budded later beyond the original growing edge.

7.3.1.3 Sexual investment

ANOVA on square root-transformed data showed no significant differences in sexual allocation between colonies at the beginning of the experiment ($F= 2.52$; $DF= 3$; $p>0.05$; for the part 1, and $F=1.80$; $DF= 3$; $p>0,05$, Fig. 4). However, at the end of 8 wk ANOVA on square root-transformed data showed significant differences in sex allocation ($F= 17.75$; $DF= 3$; $p<0.001$, and $F= 16.90$; $DF= 3$; $p<0.001$, Fig. 4). Scheffé Post Hoc test showed that in part 1 and 2 of this experiment, the high sexual allocation in colonies exposed to unfiltered allosperm suspensions was significantly different from sexual allocation recorded in colonies exposed to filtered allosperm suspensions and to the control colonies. Kruskal-Wallis test showed no significant differences in sex ratio between colonies at the beginning of the experiment ($H= 1.26$; $DF= 3$; $p>0.05$, in part 1, $H= 3.50$; $DF= 3$; $p>0.05$, in part 2; Fig. 5). By the end of the week eight sex ratio was

approximately 2 (part 1) and 2-4 times (part 2) higher in colonies exposed to unfiltered allosperm suspension than in colonies exposed to filtered allosperm suspensions and in the control colonies ($H=6.34$; $DF= 3$; $p<0.05$; part 1, and $H= 9.38$; $DF= 3$; $p<0.05$; part 2).

7.3.1.4 Colony size

Kruskal-Wallis analysis of colonial size indicate no significant differences in colony size in different treatments between the colonies at the beginning of the experiment ($H= 1.25$; $DF= 3$; $p>0.05$; in the part 1 and $F= 4.13$; $DF= 3$; $p>0.05$, in part 2; Fig. 6). Similarly, analysis of the final colonial sizes after 8 wk showed no significant differences ($H= 4.13$; $DF= 3$; $p>0.05$; in part 1, and $H=6.44$; $DF= 3$; $p>0.05$; in part 2; Fig. 6).

7.3.2 Experiment 2: effect of not biological stressors at the growing edge on sexual allocation

7.3.2.1 Physical contact

No reproductive zooids were recorded close to the growing edge of the colonies before contact took place. Interruption of colonial growth was the first response when the colonial edge made contact with the piece of glass. This result was found in the normal and slow growing genotypes. In normal growing colonies, more basal and frontal males per basal autozooids were recorded in the contact areas than in the contact-free areas and in the control colonies (Fig. 7a). Similar and low number of female zooids per basal male was recorded in the contact and contact-free areas. ANOVA on square root-transformed data on basal males showed differences attributed to treatment ($F= 24.72$; $DF= 2$; $SS= 0.63$; $MS= 0.31$; $p<0.0001$) and to the combined effect of clone and treatment ($F= 10.08$; $DF= 5$; $SS= 0.64$; $MS= 0.13$; $p<0.0001$). However, there were no significant differences attributed to clone ($F= 0.32$; $DF= 3$; $SS= 0.01$; $MS= 0.004$; $p>0.05$). ANOVA on square root-transformed frontal males data showed differences attributed to treatment ($F= 12.47$; $DF= 2$; $SS=0.27$; $MS= 0.14$; $p<0.0001$) and to the combined effect of clone and treatment ($F= 5.15$; $DF= 5$; $SS= 0.28$; $MS= 0.06$; $p<0.001$). There were no significant differences attributed to clone ($F= 0.26$; $DF= 3$; $SS= 0.008$; $MS= 0.003$; $p>0.05$). No female zooids were recorded in the control colonies (Fig. 7a), Since female zooids were not recorded in the control colonies, sex ratio was only calculated in the contact and

contact-free areas. Sex ratio was higher in the contact area than in the contact-free areas (Fig. 7b), however no significant different attributed to the combine effect of treatment and clone ($F= 0.63$; $DF= 4$; $SS= 0.47$; $MS= 0.12$; $p>0.05$), clone ($F= 0.32$; $DF= 3$; $SS= 0.18$; $MS= 0.06$; $p>0.05$) or treatment ($F= 1.57$; $DF= 1$; $SS= 0.29$; $MS= 0.29$; $p>0.05$) were found. The highest sexual allocation values were found in the contact areas (Fig. 7c). ANOVA on square root transformed sexual allocation showed differences attributed to treatment ($F= 1.37$; $DF= 2$; $SS= 1.37$; $MS= 0.69$; $p<0.0001$) and to the combined effect of clone and treatment ($F= 17.46$; $DF= 5$; $SS= 1.38$; $MS= 0.28$; $p<0.0001$). There were no significant differences attributed to clone ($F= 0.18$; $DF= 3$; $SS= 0.008$; $MS= 0.003$; $p>0.05$).

Similarly to normal growing colonies, significantly more basal males per basal autozooids were recorded in the contact area than in similar areas in the contact-free and control colonies (Fig. 7d). These differences were attributed to the interaction of treatment and clone ($F= 17.46$; $DF= 5$; $SS= 1.38$; $MS= 0.28$; $p<0.0001$) and to treatment ($F= 43.39$; $DF= 2$; $SS= 1.37$; $MS= 0.69$; $p<0.0001$). However, there were no significant differences attributed to clone ($F= 0.18$; $DF= 3$; $SS= 0.008$; $MS= 0.003$; $p>0.05$). Proportion of frontal males to basal autozooids were similar and higher in contact and contact-free areas than in the control colonies. ANOVA on square root-transformed data showed that differences were attributed to clone ($F=3.52$; $DF= 2$; $SS= 0.08$; $MS= 0.04$; $p>0.05$), treatment ($F=5.75$; $DF= 2$; $SS= 0.14$; $MS= 0.07$; $p>0.05$), and to the interaction between these variables (treatment ($F=4.64$; $DF= 4$; $SS= 0.22$; $MS= 0.05$; $p>0.05$). Scheffé post hoc test on one-way ANOVA showed that proportions of frontal males to basal autozooids in the control colonies were significantly different from those in contact and contact-free colonies ($p>0.05$). Moreover, no significant differences were detected between these two treatments ($p<0.05$).

Differing from the normal growing genotypes, female zooids were present at similar proportions to basal autozooids in the contact and contact-free areas of experimental colonies, and these proportions were similar to these on the control colonies (Fig. 7d). ANOVA on square root-transformed data showed significant differences attributed to treatment ($F= 0.02$; $DF= 2$; $SS= 0.07$; $MS= 0.04$; $p<0.05$) and to the combine effect of treatment and clone ($F= 2.75$; $DF= 4$; $SS= 0.1$; $MS= 0.03$; $p<0.05$). However, no

significant differences were attributed to clone (F= 1.50; DF= 2; SS= 0.03; MS= 0.01; p<0.05).). Scheffé post hoc test showed that proportions of female zooids to basal autozooids in the contact areas of colonies were not significantly different from areas of contact-free colonies (p>0.05), but were significantly different from the control colonies (p<0.05). As a consequence of the highest number of basal males occurring in the contact areas, sex ratios in these areas were lowest than in the contact-free and control colonies (Fig. 7e). However, ANOVA on square root-transformed data showed no significant differences attributed to treatment (F= 0.17; DF= 2; SS= 0.16; MS= 0.08; p>0.05), clone (F= 0.33; DF= 2; SS= 0.32; MS= 0.16; p>0.05) or to the interaction of these two variables (F= 0.26; DF= 4; SS= 0.49; MS= 0.12; p>0.05). Sexual allocation was higher in contact areas than in contact-free and control colonies (Fig. 7f). ANOVA on square root-transformed data showed significant differences attributed to treatment (F= 25.08; DF= 2; SS= 0.39; MS= 0.19; p<0.0001) and to the combined effect of treatment and clone (F= 12.79; DF= 4; SS= 0.40; MS= 0.10; p<0.0001). However, no significant differences attributed to clone were detected (F=0.51; DF= 2; SS= 0.008; MS= 0.004; p>0.05). Scheffé post hoc test on one-way ANOVA showed that sexual allocation on the contact areas was significantly higher than in the contact-free areas and control colonies.

In the contact area, dwarf basal autozooids were common. ANOVA on basal area of basal autozooids showed significant differences in basal zooid areas among treatments (F=7.22; DF= 2; p<0.001). Scheffé post hoc test showed no significant differences in basal autozooid area among contact-free and control. However, significant differences were found between these basal autozooids and basal autozooids from the contact area (Table 1).

7.3.2.2 Biological contact

As in the no biological contact, interruption of colonial growth was the first response found when two colonies made contact by their growing edges. This result was found in both iso and allocontact with normal and slow growing clones. Moreover, in the contact area erect growth of one colony using the other colony as substratum and partial overgrowth were also found. Colonial fusion, evidenced by the presence of coalescent

zooids, was only found in isocontacts. Basal males were found along the contact area of the iso and allocontact, however coalescent basal males were only found in isocontact. Presence of dwarf basal autozooids was also common in the contact area between iso- and allocontact of normally and slowly growing clones. Significant differences were detected when the basal area of the autozooids were compared (Table 2), autozooids in the contact area being smaller than in the contact-free and control areas.

7.3.2.2.1. Isocontact

7.3.2.2. 1.1 Normally growing clones

No reproductive zooids were recorded close to the growing edge of the colonies before the contact between the colonies took place. After contact, basal males were the only reproductive zooids recorded in the contact area. No females and frontal males were recorded in this area (Fig. 8a). Since no females were produced in these colonies, values of sex ratio were zero (Fig. 8b), however sexual allocation reached higher values in the contact areas than in the free areas (Fig. 8c).

7.3.2.2.1.2 Slowly growing clones

Before contact between the colonies, sexual zooids were recorded close to the growing edges. After contact, presence of basal males was recorded only in the contact area (Fig 8a). No basal males were recorded in the contact-free area of the same colonies or in the control colonies (Fig. 8a). Differing from the normal growing colonies, frontal males and female zooids were recorded in the contact area, in the contact-free area and in the control colonies (Figs. 8d). However, no significant differences in proportion of frontal males to basal autozooids were attributed to treatment ($F= 0.57$; $DF= 2$; $SS= 0.02$; $MS= 0.01$; $p>0.05$), clone ($F=1.05$; $DF= 3$; $SS= 0.06$; $MS= 0.02$; $p>0.05$) or the combined effect of these two variables ($F= 0.86$; $DF= 5$; $SS=0.08$; $MS= 0.02$; $p>0.05$). Similarly, no differences in proportion of female zooids to basal autozooids were attributed to treatment ($F= 0.96$; $DF= 2$; $SS= 0.04$; $MS= 0.02$; $p>0.05$), clone ($F=0.77$; $DF= 3$; $SS= 0.05$; $MS= 0.02$; $p>0.05$) or the combined effect of these two variables ($F= 0.85$; $DF= 5$; $SS= 0.08$; $MS= 0.02$; $p>0.05$). Although sex ratio was higher in the control colonies than in the both contact and contact-free areas (Fig. 8e), ANOVA on square root transformed sex ratio showed no significant differences attributed to treatment ($F= 0.74$;

DF= 2; SS= 0.38; MS= 0.19; p>0.05), clone (F=0.54; DF= 3; SS= 0.42; MS= 0.14; p>0.05) or the combined effect of these two variables (F= 0.62; DF= 5; SS= 0.80; MS= 0.16; p>0.05). However, ANOVA on square root transformed sexual allocation showed significant differences attributed to treatment (F= 10.60; DF= 2; SS= 0.32; MS= 0.16; p<0.0001) and the combined effect of treatment and clone (F= 5.06; DF= 5; SS= 0.38; MS=0.08; p<0.001). No significant differences were attributed to clone (F= 1.37; DF= 3; SS= 0.06; MS= 0.02; p>0.05). Scheffé post hoc test showed that significantly higher sexual allocation values were associated with the contact areas than in contact-free and control colonies (p<0.05, Fig. 8f).

7.3.2.2.2 Allocontact

7.3.2.2.2.1 Normally growing clones

In normally growing colonies, basal males were only recorded in the contact area. Differing from the isocontact, frontal male zooids and female zooids were also recorded (Fig. 9a). Proportions of frontal males and female zooids to basal autozooids were similar between contact, contact-free and control colonies. ANOVA on square root-transformed proportion of frontal male zooids to basal autozooids showed no significant differences attributed to treatment (F= 2.16; DF= 2; SS= 0.06; MS= 0.03; p>0.05), clone (F= 1.87; DF= 3; SS= 0.08; MS= 0.03; p>0.05) or the combined effect of these two variables (F= 1.98; DF= 5; SS= 0.14; MS= 0.03; p>0.05). Similarly, ANOVA on square root-transformed proportion of female zooids to basal autozooids showed no significant differences attributed to treatment (F= 2.24; DF= 2; SS= 0.06; MS= 0.03; p>0.05), clone (F= 1.21; DF= 3; SS= 0.05; MS= 0.02; p>0.05) or the combined effect of these two variables (F= 1.63; DF= 5; SS=0.11; MS= 0.02; p>0.05). Although sex ratio was higher in control colonies than in contact and contact-free colonies (Fig. 9b), ANOVA on square root-transformed data showed no significant differences attributed to treatment (F= 1.95; DF= 2; SS= 0.96; MS= 0.48; p>0.05), clone (F= 0.42; DF= 3; SS= 0.31; MS= 0.10; p>0.05) or the combined effect of these two variables (F= 1.03; DF= 5; SS=0.27; MS= 0.25; p>0.05). However, sexual allocation was higher in the contact areas than in the contact-free and control colonies (Fig. 9 c), ANOVA on square root-transformed data showed significant differences attributed to treatment (F= 10.90; DF= 2; SS= 0.20; MS= 0.10; p<0.0001) and the combined effect of treatment and clone (F= 4.46; DF= 5;

SS= 0.21; MS= 0.04; p>0.05). However, no significant differences attributed to clone were detected (F=0.16; DF= 3; SS= 0.004; MS= 0.002; p>0.05). Scheffé post hoc test showed significantly lower sexual allocation in the control colonies and contact-free areas than in contact areas (p<0.05, Fig. 9c).

7.3.2.2.2.2 Slowly growing clones

In slowly growing colonies, basal males were only recorded in the contact area. As in the normal growing allocontact, frontal male zooids and female zooids were also recorded (Fig. 9a). ANOVA on square root transformed proportions of frontal males to basal autozooids showed not significant differences attributed to treatment (F= 2.28; DF= 2; SS= 0.11; MS= 0.05; p>0.05), clone (F= 0.96; DF= 3; SS= 0.04; MS= 0.01; p>0.05) or the combined effect of these two variables (F= 2.29; DF= 5; SS= 0.14; MS= 0.03; p>0.05). ANOVA on square root-transformed proportions of female zooids to basal autozooids showed significant differences attributed to clone (F= 5.47; DF= 3; SS= 0.16; MS= 0.05; p<0.05) and the interaction of clone and treatment (F= 4.14; DF= 5; SS= 0.20; MS= 0.04; p<0.05). No significant effect of treatment on the proportion of female zooids to basal zooids was found (F=2.16; SS= 0.05; MS=0.02; p>0.05). Despite basal males being recorded only in the contact areas, ANOVA on square root-transformed sex ratio showed no significant differences between contact, contact-free and control colonies attributed to treatment (F= 0.12; DF= 2; SS= 0.07; MS= 0.04; p>0.05), clone (F= 2.53; DF= 3; SS= 2.32; MS= 0.77; p>0.05) or the combined effect of these two variables (F= 1.56; DF= 5; SS= 2.39; MS= 0.48; p>0.05, Fig 9 b). However, ANOVA on square root-transformed sexual allocation showed significant effects of treatment (F= 5.44; DF= 2; SS= 0.63; MS= 0.03; p<0.05), clone (F= 4.5; DF= 3; SS= 0.08; MS= 0.03; p<0.05) and the combined effect of these two variables (F= 4.88; DF= 5; SS= 0.14; MS= 0.03; p<0.001). Scheffé post hoc test in a one-way ANOVA showed significantly lower sexual allocation in the control colonies than in contact and contact-free areas (p<0.05, Fig. 9f).

7.3.3 Experiment 3: effect of not biological disturbance at the colonies on sexual allocation

7.3.3.1 Temperature

The first clear consequence of thermal shock was the diminution in the proportion of active zooids in the colonies. Before the thermal shock, the proportion of active zooids in the colonies ranged from 0.65 and 0.70 in the control colonies and from 0.67 and 0.71 in the stressed colonies. However, after the thermal shock the proportion of active zooids in the stressed colonies diminished to close to zero. Basal males were only recorded in the stressed colonies (Fig. 10 a). Basal males in the stressed colonies were budded contiguously forming a ring of basal males (see Chapter 8, Plate 3). Proportions of frontal male zooids to basal autozooids were low and not significantly different between stressed and control colonies ($H= 0.008$; $DF= 1$; $p>0.05$, Fig. 10a). Female zooids were not recorded within the sampling area in the stressed and in the control colonies (Fig. 10 a), and sex ratio could not be calculated (Fig. 10 b). However, ANOVA on square root-transformed sex allocation showed significant differences attributed to treatment ($F= 57.40$; $DF= 1$; $SS= 0.86$; $MS= 0.86$; $p<0.0001$) and the combined effect of treatment and clone ($F= 14.63$; $DF= 4$; $SS= 0.88$; $MS= 0.22$; $p<0.0001$). High values of sexual allocation corresponded to stressed colonies (Fig. 10c) No differences attributed to clone were detected ($F= 0.38$; $DF= 3$; $SS= 0.02$; $MS= 0.006$; $p>0.05$).

Dwarf basal autozooids were budded beyond the original growing edge of the stressed colonies. Significant differences in the area of the basal autozooids between the stressed areas and the control ones were detected (Table 3).

7.3.3.2 Desiccation

As in the previous experiment, the first clear consequence of desiccation was the diminution on the proportion of active zooids in the stressed colonies. However, some of the active zooids became recycling zooids and others were killed after the stress was applied. Killed zooids were evidenced by the red pigments of digested cells of R. reticulata leaking from the damaged zooidal gut. Eventually, some of these zooids were able later to produce new functional polypides, however with a lower turnover in comparison with recycling zooids (personal observation). As with the previous stressor, basal males were

only recorded in the stressed colonies (Fig. 11a). Frontal males were recorded in stressed and control colonies, with a higher proportion of frontal males to basal autozooids being found in the control colonies (Fig 11. a). ANOVA on square root-transformed data showed significant differences only attributed to treatment (F= 6.43; DF= 1, SS= 0.05; MS= 0.05; $p<0.05$). However, no significant effects of clone (F= 0.46; DF= 3; SS= 0.01; MS= 0.004; $p>0.05$) and the combined effect of treatment and clone were detected (F= 1.95; DF= 4; SS= 0.07; MS= 0.02; $p>0.05$). Sex ratio could only be computed in the control colonies, which reached a mean value about 0.3 (Fig. 11 b). Sexual allocation was very similar in the control colonies than in the stressed (H=2.24; DF= 1; $p>0.05$, Fig. 11c). As with in the previous stressors, dwarf basal autozooids were budded beyond the original growing edge of the stressed colonies. Again area of the basal autozooids was significantly smaller in stressed colonies than in the controls (Table 3).

7.3.3.3 Food availability

As with the previous stressors, privation of food also produced a significant reduction in the proportion of active zooids. No basal males were present in the colonies at the beginning of the experiment. However, two months after the stressor was applied presence of basal male zooids was recorded only in stressed colonies. Frontal male zooids were recorded in control and stressed colonies and females zooids only in the control colonies (Fig. 12 a). ANOVA on square root transformed proportion of basal males to basal autozooids showed no significant differences attributed to treatment (F= 3.03; DF= 1; SS= 0.02; MS= 0.02; $p>0.05$), clone (F= 0.15; DF= 3; SS= 0.003; MS= 0.001; $p>0.05$) and to the combined effect of these two variables (F= 0.88; DF= 4; SS= 0.03; MS= 0.007; $p>0.05$). Sex ratio could be computed only for control colonies, reaching a mean value of about 0.2 (Fig. 12 b). Kruskal-Wallis test showed no significant differences in sex allocation between stressed and control colonies (H= 0.001; DF= 1; $p>0.05$, Fig. 12 c).

As with the two previous stressors, the first basal zooids budded beyond the original growing edge were smaller than the previous ones. Significant differences on the basal area of these zooids in comparison with equivalent autozooids in control colonies were detected Significantly smaller basal autozooids were found in stressed colonies than in the controls (Table 3).

7.3.3.4 Mechanical damage:

The first zooids recorded in the damaged areas at the original growing edge of the colonies (Treatment 1) and apart from the original growing edge of the colonies (Treatment 2) were basal autozooids. However, basal males were also recorded in the stressed areas but not in similar areas in the control colonies (Fig. 13 a and d). Frontal males were also recorded in the stressed and control areas. However, the proportion of frontal males to basal autozooids was approximately 5 times higher in the damaged areas apart from the original growing edge of the colonies (Treatment 2) than in areas at the growing edge (Treatment 1). Kruskal-Wallis test showed no significant differences in the proportion of frontal males to basal autozooids between areas at the growing edge and control colonies ($H= 0.037$; $DF= 1$; $p>0.05$, Fig. 13 a). In areas apart from the original growing edge more frontal males to basal autozooids were recorded in the control colonies than in the stressed colonies (Fig. 13.d). ANOVA on square root transformed proportion of frontal males to basal males showed significant differences attributed to treatment ($F= 11.89$; $DF= 1$; $SS= 0.20$; $MS= 0.20$; $p< 0.001$), clone ($F= 3.75$; $DF= 3$; $SS= 0.19$, $MS= 0.06$; $p<0.05$) and to the combined effect of these two variables ($F= 5.79$; $DF= 4$; $SS= 0.38$; $MS= 0.01$; $P<0.0001$).

In the damaged areas at to the growing edge, female zooids were not recorded. However, they were recorded in equivalent areas in the control colonies (Fig. 13 a). In these colonies sex ratio was about 0.1 (Fig 13. b). A Kruskal-Wallis test showed that sexual allocation was significantly higher in areas at the growing edge than in equivalent areas in the control colonies ($H= 17.96$; $DF= 1$; $p<0.0001$, Fig. 13 c).

Female zooids were recorded in damaged areas close to the original growing edge and in equivalent control areas (Fig. 13 d). Higher proportions of female zooids to basal autozooids were recorded in the control areas than in the damaged areas. A Kruskal-Wallis test showed significant differences in between these areas ($H= 17.95$; $DF= 1$; $p<0.0001$). Despite sex ratio being higher in the damaged areas than in the control ones (Fig. 13 e), ANOVA on square root-transformed data showed no significant differences attributed to treatment ($F= 1.45$; $DF= 1$; $SS= 0.33$; $MS= 0.33$; $p>0.05$), clone ($F= 0.92$; $DF= 3$; $SS= 0.62$; $MS= 0.21$; $p>0.05$) and to the combined effect of these two variables clone ($F= 1.05$; $DF= 4$; $SS= 0.95$; $MS= 0.24$; $p>0.05$). However, sexual allocation in

similar areas of the control colonies was significantly higher than in the damaged areas (H= 17.48; DF= 1; p<0.0001, Fig. 13f).

The first basal autozooids budded in the damaged areas were budded from the rest of the destroyed zooecium. This was recorded in mechanically damaged localized areas at the growing edge as well as within the colonies. When the mechanical damage was localized within the basal autozooids were budded in opposite direction to the growing pattern (i.e. centripetally. Significant differences were detected between the area of these basal zooids and equivalent basal zooids in control colonies. Significantly smaller basal autozooids were found in stressed colonies than in the controls (Table 4).

7.3.4 Experiment 4: effect of exposure to sperm suspensions of different genetic relatedness on sexual allocation and fertilization success.

Colony size at the beginning of the experiment ranged between 100 and 140 mm^2 (Fig. 14. a). A Kruskal-Wallis test showed no significant differences in colony size between the colonies at the beginning of the experiment (H= 3.15; DF= 4; p>0.05). Similarly, at the end of the experiment, 2 mo later, a Kruskal-Wallis test showed no significant differences in colony size between the colonies (H= 1.05; DF= 4; p>0.05, Fig. 14 a). Presence of progeny was recorded in all the colonies exposed to unrelated and half- sib sperm suspensions. However, progeny were recorded only in 33% (N=6) of the colonies exposed to full-sib sperm suspensions and in 17% (N=6) of the colonies exposed to maternal sperm . No progeny were produced by the control colonies exposed to selfsperm suspensions (Fig. 14 b). ANOVA on the reproductive output, i.e. ratio of embryos produced per colony to number of female zooids, showed significant differences attributed to the treatment, i.e. genetic relatedness between the receptor colonies and the sperm to which the receptor colonies were exposed (F= 30.17; DF= 4; SS= 0.55; MS= 0.14; p<0.0001) and the combined effect of treatment and clone (F= 14.53; DF= 9; SS= 0.60; MS= 0.07; p<0.0001). However, no differences attributed to clone were detected (F= 2.02; DF= 5; SS= 0.05; MS= 0.01; p>0.05). Sex ratio at the beginning of the experiment ranged between 0.6 and 0.8, and no significant differences between the treatment were detected (H= 0.17 ; DF= 4; p>0.05, Fig.14 c). At the end of the experiment sex ratio values for colonies exposed to unrelated and half-sib sperm suspensions were about 2, i.e. twice the sex ratio recorded in colonies exposed to self, full-sib and maternal sperm

suspensions (Fig. 14 c). ANOVA on sex ratio showed significant differences attributed to treatment (F= 18.18; DF= 1.27; SS= 5.09; MS= 1.27; p<0.0001) and to the combined effect of treatment and genotype of the receptor clones (F= 8.66; DF= 9; SS= 5.45; MS= 0.61; p<0.0001). No significant differences attributed to clone were detected (F= 1.03; DF= 5; SS= 0.36; MS= 0.07; p>0.05). Scheffé post hoc tests showed no significant differences between sex ratio in colonies exposed to half-sib and unrelated sperm suspensions, but these were significantly higher than sex ratios in colonies exposed to self, full-sib and maternal sperm suspensions (p>0.05, Fig. 14. c). The same test showed no significant differences in sex ratio among colonies exposed to self, full-sib and maternal sperm suspensions (p<0.05). No significant differences were found in sexual allocation at the beginning of the experiment (H: 0.17; DF= 4; p>0.05). However, 2 mo later at the end of the experiment significant differences in sexual allocation were found (H= 9.11; DF= 4; p<0.05, Fig. 14 d). The highest sexual allocation was associated with colonies exposed to half-sib and unrelated sperm suspensions.

7.3.5 Experiment 5: effect of continuous exposure to allosperm on the onset of sexual reproduction and sexual allocation.

7.3.5.1 From 10 basal autozooids

7.3.5.1.1 Colony size, proportion of active basal zooids and index of circularity

No significant differences were found in colony size at the beginning of parts 1 and 2 of the experiment (H: 0.15; DF= 3.8; p>0.05; for the part 1, H=0.58; DF= 2; p>0.05; for the part 2, Fig 15 a and b). However, after 10 weeks significant differences were detected, with colonies exposed daily to allosperm significantly smaller than the others (H= 11.57; DF= 2; p<0.05; for the part 1, H= 10.33; DF= 2; p<0.001; for the part 2, Fig 15 a and b, Plate 2). During the entire experimental period the percentage of active zooids in the colonies was over 70 % (Fig. 16 a and b) and the index of circularity (see Appendix 1 for description of the index) tended asymptotically to the maximum value of 1 corresponding to circular colonies (Fig. 17 a and b).

7.3.5.1.2 Onset of sexual reproduction

The timing and colony size when reproductive zooids were first recorded under the experimental conditions are summarized in the Table 5. In part 1 and 2 of the present

experiment, the first complete sexual zooids were recorded by the end of the first week. These sexual zooids corresponded to basal males budded mainly in association with the damaged areas in the colonies. In some colonies the first basal male zooids were recorded only when the colonies had grown and contact between growing edges of the same colony took place. However, on one occasion (Treatment 1, part 1) after 10 wk no basal males were recorded.

The first complete frontal males were recorded from the end of the first week to the end of the second week when the average colony size ranged from 33 to 86 basal autozooids (Table 5). However, sperm activity inside these zooids was only recorded one week after the male zooids were formed (personal observations). The first incipient female zooids were recorded from the first week to the beginning of the second week when the average colony size ranged from 26 to 56 basal autozooids (Table 5). In the part 1 of the experiment, incipient female zooids in control colonies appeared in all the colonies by the third week. However, in colonies exposed only during the first day to allosperm suspensions (Treatment 1) or with daily allosperm exposure (Treatment 2) presence of incipient female zooids appeared by the first week. Similarly, in the part 2 presence of incipient female zooids was achieved by the third week in the control colonies and one week before in the Treatment 1 and Treatment 2. The first developing oocytes inside the coelom of incipient female zooids were recorded when the colony size ranged from 24 to 30 basal autozooids. These oocytes were only recorded in colonies exposed to allosperm suspensions (see below).

In the part 1, the first complete female zooids appeared in all the colonies by the second wk in the Treatment 1 and Treatment 2. By this time the colonies were about 60 basal autozooids in size (Table 5). However, in the control colonies complete female zooids appeared only one wk later (Table 5). In the part 2, the first complete female zooids appeared achieved in all the colonies after 1 wk in the Treatment 2. However, in the Treatment 2 and in the control colonies complete female zooids appeared in all the colonies approximately by the second and third wk respectively.

7.3.5.1.3 Sexual allocation and sex ratio

Sexual allocation and sex ratio throughout the 10 experimental weeks are summarized in the Figure 18 and Figure 19. Sex allocation increased from the beginning of the experiment in all the colonies including the controls. At the end of the experiment, significant differences were detected, the highest sex allocation being associated with colonies from Treatment 2 in part 1 and 2 ($H= 13.66$; $DF= 2$; $p<0.001$; in part 1, $H=15.17$; $DF= 2$; $p<0.001$; in part 2, Fig. 18 a and b). Sex allocation in colonies from Treatment 1 and in the control colonies was maintained at similar and low levels during the entire experimental period. Higher sex ratios were recorded in colonies from treatment 1 and 2. However, by the fifth week sex ratio in colonies from Treatment 1 started to decreased, reaching values close to unity by the end of the experiment (Fig. 19 a and b). By the end of the experiment, significant differences in sex ratio were detected ($H= 13.80$; $DF= 2$; $p<0.001$; in part 1; $H=11.79$; $DF= 2$; $p<0.005$; in part 2).

7.3.5.1.4 Reproductive output

Oocytes, embryos and viable ancestrulae were only produced in colonies exposed to allosperm (Treatment 1 and 2). No progeny were recorded in the control colonies. In colonies exposed to allosperm only during the first day (Treatment 1), part 1, a small peak in the proportion of female zooids bore progeny by the second week. However, after that the proportion diminished reaching zero by the seventh week (Fig. 20 a). In colonies from Treatment 1, part 2, maximum reproductive output was reached by the third week. By the eighth week reproductive output diminished to zero in both parts of the experiment (Fig. 20 b). In colonies exposed daily to allosperm (Treatment 2), part 1, from the third week onwards reproductive output was about 0.4 (Fig. 20 a). However, in part 2, from the third week onwards reproductive output ranged from 0.6 to 0.8 (Fig. 20 b).

7.3.5.2 From ancestrulae

7.3.5.2.1 Colony size, proportion of active basal zooids and index of circularity

No significant differences in colony size were detected at the beginning of the experiments ($H= 1.10$; $DF= 2$; $p>0.05$; in part 1 $H= 1.04$; $DF= 2$; $p>0.05$; in part 2). However, at the end of the experiment significant differences were found ($H= 7.73$; $DF= 2$; $p<0.05$; in part 1, $H= 7.39$; $DF= 2$; $p<0.05$; in part 2). Small colony size was associated with exposure to allosperm continuously from the beginning of the experiment (Fig. 21 a and b).

Throughout the entire experimental period, the proportion of active basal zooids never was lower than 70% (Fig. 22 a and b), and high indices of circularity were achieved by the fourth week (Fig. 23 a and b).

7.3.5.2.2 Onset of sexual reproduction

The timing and colony size when reproductive zooids were recorded under the experimental conditions are summarized in the Table 6. In contrast to the previous experiment starting from 10 zooid-sized colonies, in the present experiment the first sexual zooids were not always basal males. The appearance of basal males coincided with contact between the growing edge within the same colony, and in some colonies basal males were not recorded during the entire experimental period. The first sexual zooids achieved in all the experimental colonies were incipient female zooids and frontal males zooids. Frontal males were recorded in all the colonies assigned to both the experimental treatments and to the controls (Table 6). In part 1 and 2, presence of frontal males and incipient female zooids was achieved in all the colonies of Treatment 1 and Treatment 2 by the third week, however in control colonies this condition was achieved only 1 wk later. Presence of complete female zooids in all the colonies was recorded by the third (part 1) and fourth week (part 2) in colonies from Treatment 2. However, in the control colonies and colonies from Treatment 1 the same condition was recorded by the fourth and fifth week respectively.

Sperm activity was recorded inside the female zooids about 1 wk after the zooids were completed. However, oocytes were recorded inside the coelom of incipient females when colony size ranged from 20 to 30 basal zooids.

7.3.5.2.3 Sexual allocation and sex ratio

Sexual allocation and sex ratios throughout the 10 experimental weeks are summarized in Figure 24 and Figure 25. Sex allocation increased from the third week onwards in the control colonies and Treatment 1 and Treatment 2. However, by the end of the experiment sex allocation was significantly different ($H= 7.42$; $DF= 2$; $p<0.05$; in part 1, $H= 7.54$; $DF= 2$; $p<0.05$; in part 2). Similarly, sex ratio increased from the third week onwards reaching maximum values in colonies from Treatment 2. In Treatment 1 a maximum peak of sex ratio was reached by the fourth week and after that sex ratio diminished reaching

values close to 1. In control colonies during the entire experimental period sex ratio was maintained close to 1 (Fig. 25 a and b). By the end of the experiment, significant differences in sex ratio were detected ($H= 7.42$; $DF= 2$; $p<0.05$; in part 1, $H= 8.58$; $DF= 2$; $p<0.05$; in part 2).

7.3.5.2.4 Reproductive output

As in the experiment with 10 zooid colonies, in the present experiment oocytes, embryos and viable ancestrulae were only produced in colonies exposed to allosperm (Treatment 1 and 2). No progeny were recorded in the control colonies. Reproductive output was very low in colonies from Treatment 1, reaching maximum values of 0.1 and 0.05 by the fourth week in part 1 and 2 respectively (Fig. 26 a and b). By the eighth week reproductive output decreased to zero in both parts of the experiment (Fig. 26 a and b). However, in colonies from Treatment 2 reproductive output was maintained at about 0.6 from the fifth week onwards (Fig. 26 a and b).

7.4 Discussion

7.4.1 Oocyte maturation

Presence of oocytes only in colonies exposed to unfiltered allosperm showed clearly that presence of allosperm has an important role in triggering egg production and oocyte maturation (Experiment 1). Similar results have been recorded in Diplosoma listerianum (JDD Bishop, personal communication), a colonial marine ascidian with a similar mating strategy. Moreover, presence of viable progeny only in colonies exposed to allosperm suspensions are in agreement with other experiments in this thesis indicating prevalence of outcrossing in C. hyalina.

7.4.2 Reproductive allocation

In C. hyalina variations in sexual investment have been demonstrated previously (Cancino and Hughes. 1987; DJ. Hughes, 1989; Hunter and Hughes, 1995). Thus, phenotypic adjustment of reproductive investment depending on environmental condition could play an important role in the life history of organisms living in a variable environment. Moreover, higher sex ratio and sexual allocation recorded in the presence of unrelated and half-sib sperm suspensions (Experiment 4) suggest that modulation of reproductive allocation due to presence of neighbours depend on genetic relatedness of the potential mates. Moreover, production of progeny mainly in colonies exposed to unrelated and half-sib colonies (Experiment 4) suggests that genetic relatedness has a strong effect on fertilization success. Absence of progeny in control colonies is in agreement with similar experiments in this thesis showing prevalence of outcrossing.

Significant effects in sex allocation and sex ratio supported by results of the present study (Experiment 1, Experiment 4 and Experiment 5) suggest that the presence of sexually compatible sperm could modulate production of sexual zooids. Thus production of mainly female zooids, could be delayed until presence of allosperm is assured. Significantly more female zooids have been recorded in colonies of C. hyalina growing in the field under unrestricted flow conditions (Cancino and Hughes, 1987). If water flow is related to food availability, highest female investment can be achieved when more resources are available. In the present study percentage of active zooids was always exceeded 60% and food was

always provided, therefore no differences in sexual investment due to these variables are expected. However, it is also possible that unrestricted water flow allows access to more sperm than restricted flow. This is supported by slightly more female zooids per male zooids found by Cancino (1983) at high colonial densities.

7.4.3 Onset of sexual reproduction

The presence of sexual zooids in control colonies and in colonies exposed to filtered allosperm suspensions suggests that the presence of allosperm is not necessary for the onset of production of sexual zooids (Experiment 1, Experiment 5). These results are not in agreement with findings in the Chilean C. hyalina, were female zooids were only recorded in presence of conspecific neighbours (Cancino et al., 1991).

Colony size at first sexual maturity in some bryozoans has been summarized in Hayward and Ryland (1975). Although they noted that many authors failed to define maturity, they conclude that a minimum number of feeding zooids are required to support breeding. The minimum number of zooids required at first sexual maturity in the species reviewed by Hayward and Ryland (1975) ranged from 56 to 1500. Moreover, in Alcyonidium hirsutum, the species on which Hayward and Ryland worked, most extensively, the minimum colonial sizes at first sexual maturity are between 40 to 55 basal zooids. In C. hyalina the minimum size at first sexual maturity has been reported to be about 100 autozooids (Cancino, 1983).

However, the results of the present study showed that production of male zooids with actively swimming sperm and the presence of incipient female zooids bearing visible oocytes could be achieved at smaller colony sizes, of only 30 basal autozooids. Moreover these findings are in agreement with observations of colonies growing on their natural substratum were sexual maturity (i.e. presence of males with sperm and female zooids with oocytes) was found in colonies ranging between 22-35 basal autozooids (personal observation). Based in field experiments Cancino (1983) argued that it is not expected that C. hyalina will delay the onset of sexual reproduction. This corroborates the findings of the present study (Experiment 5). Differences between these findings and those reported by Cancino (1983) could be explained by differences in the temperature at which colonies were maintained. High temperature accelerates the onset of sexual maturity (Fromont,

1994). The onset of sexual reproduction reported by Cancino (1983) corresponded to a generation of colonies settled in October. From this time onward the water temperature at the Menai Strait ranged between 12 and 6°C (Cancino, 1983). In contrast, in the present study the colonies were maintained throughout the experimental period at temperatures about 16°C. On the other hand, observations on colonies growing on their natural substratum (personal observation) were conducted during May and June when the water temperature in the Menai Strait is about 12-14°C (Cancino, 1983).

C. hyalina has been described as a protandric species, i.e. production of males first and females later (Cancino, 1983). However, the results of the present study (Experiment 5) show that incipient female zooids bearing growing oocytes can appear before male zooids bearing active sperm. These results are in agreement with observations on colonies growing on their natural substratum (see Plate 3). In these observations colonies of 20-30 basal autozooids were recorded bearing only complete and incipient female zooids, or combined with frontal male zooids. However, presence of oocytes was recorded in these female zooids before sperm activity inside the male zooids was observed.

7.4.4 Sexual reproduction and colonial growth

Absence of negative effects on final colony size in colonies exposed to initial pulses of allosperm suspension suggests no energetic constraint between sexual reproduction and growth (Experiment 1 and Experiment 4). However, smaller sizes found in colonies exposed daily to allosperm (Experiment 5), suggests that these colonies were allocating more resources into sexual reproduction, so having less resources to allocate to growth than colonies maintained in reproductive isolation.

7.4.5 Production of basal males

Elevated allocation to production of basal males in C. hyalina has been described in association with elevated high food concentration and lower temperatures (Hunter and Hughes, 1994; 1995). In agreement with Hunter and Hughes results, in the present study production of basal males was exclusively concentrated in stressed areas of the colonies (Experiment 2 and Experiment 3). However, presence of basal males in colonies growing under optimal conditions in Hunter and Hughes study are not in agreement with

total absence of basal males in control colonies (i.e. maintained without stressors) in the present study. The phenotypic response of high production of basal males following a stress could be interpreted as a mechanism evolved to assure cross-fertilization through sperm production under environmental conditions adverse to female production and brooding.

Observations of live colonies of C. hyalina growing in their natural substratum have detected zero or low allocation to basal males (personal observation). However, groups of basal males were observed in the contact area of neighbour colonies where the colonial growth was constrained by other colonies. These results are in agreement with findings of the present study where basal males were produced in the contact area with physical and biological barriers. It has been assumed in the literature that production of sperm needs less resources than production of eggs (Cohen, 1973). This seems to be in agreement with production of basal males after colonies were subjected to stress (Experiment 3), in contrast with control colonies where basal males were not recorded. Since after the stressors were applied low percentages of active zooids were present in the colonies (ca. <10), it is suggested that basal male production could be an adaptive mechanism to assure the broadcast of gametes of low cost under deleterious conditions, where the probability of death of the genotype is high.

7.4.6 Basal autozooid size:

Morphological changes producing miniaturization of zooids as a consequence of extreme flow conditions have been described in Membranipora membranacea (Okamura and Partridge, 1999). These results are in agreement with small basal areas of the first autozooids budded after stressors were applied (Experiment 2 and Experiment 3). Although no data on lophophore size were considered in these experiments, it is possible that reduction in zooid side had implications for lophophore size. Thus less energy should be allocated to growth, budding smaller but functional zooids during the time stressful conditions are present.

Plate 1: Experimental set up. Groups of three glass vessels, each bearing a colony of C. hyalina, which were exposed to the 4 experimental treatments.

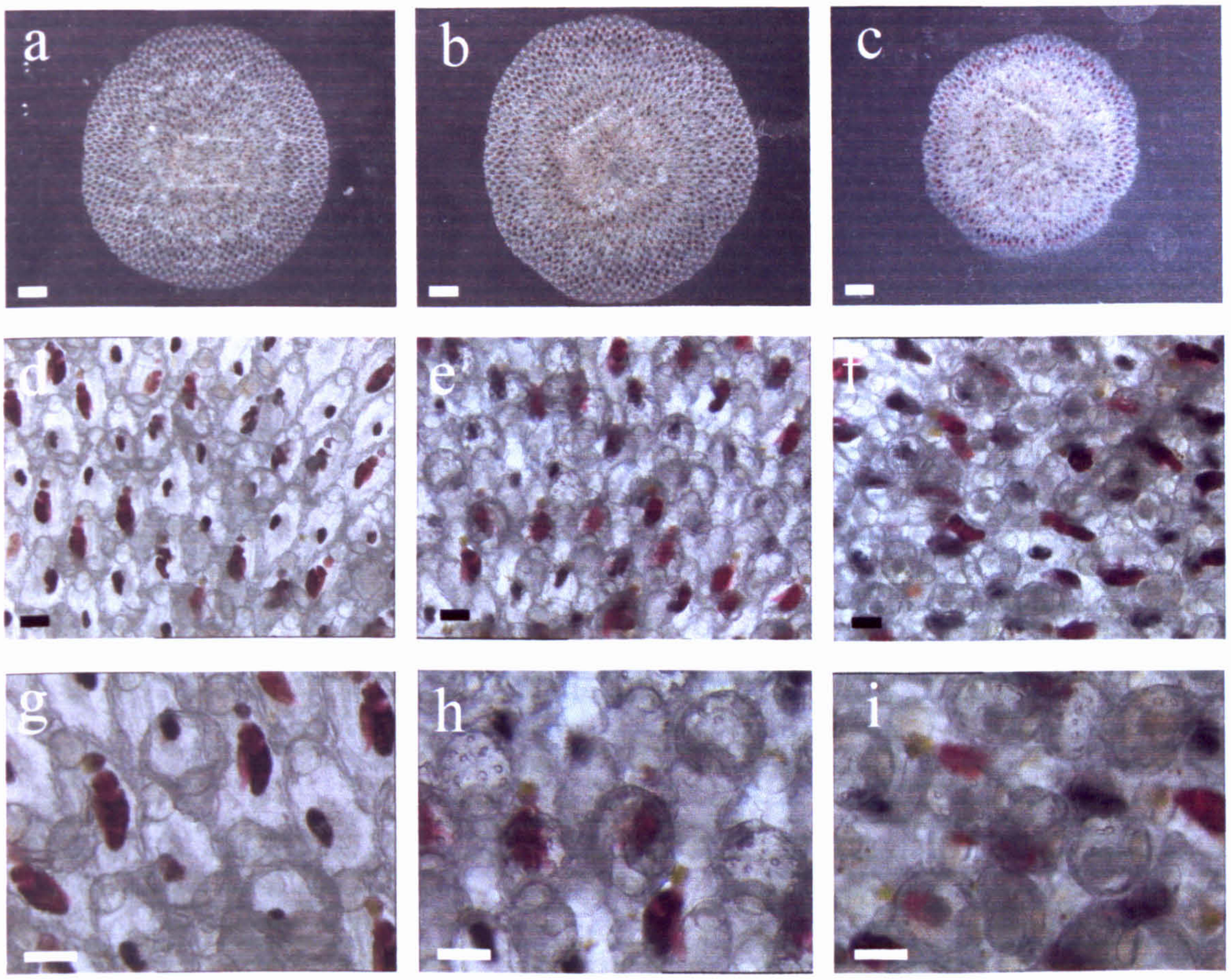

Plate 2: Experiment 5, colonies of C. hyalina maintained under three experimental conditions of exposure to allosperm suspensions at 10 basal autozooids. Views (a), (d) and (g) correspond to a control colony not exposed to allosperm suspensions; (b), (e) and (h) correspond to a colony exposed to allosperm only the first day; and (c), (f) and (i) to a colony exposed to allosperm daily. Note that pictures were taken by the end of the experiment (10 wk) and that in the control colony female zooids are mainly incipient (g, without a complete ovicell), but in colonies exposed to allosperm they are full females (h and i, with a complete ovicell). By this time embryos were only present in colonies exposed daily to allosperm suspensions. Scale bars are 1 mm in (a), (b) and (c); 100 μm in (d) to (i).

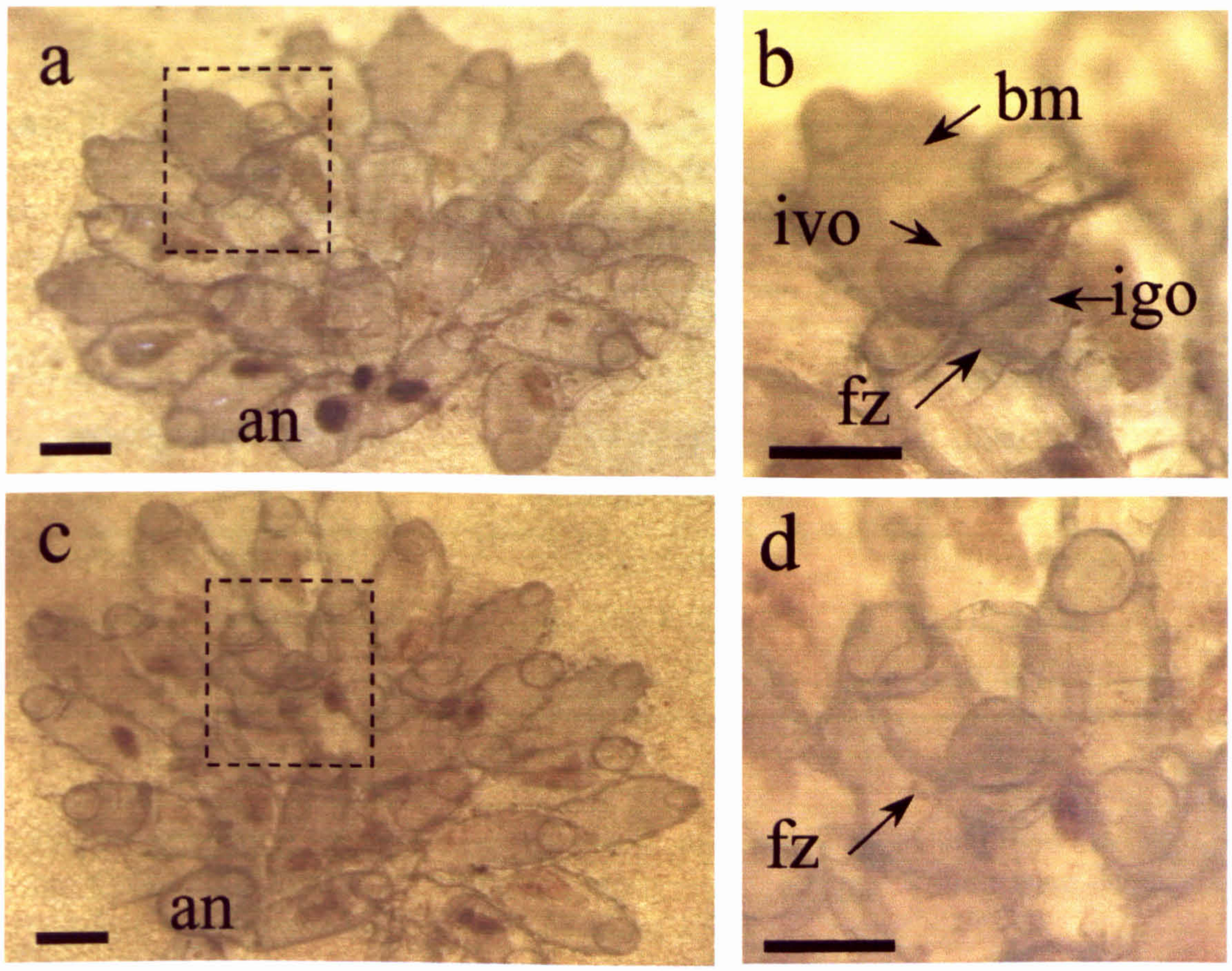

Plate 3: Views of C. hyalina colonies on their natural substrata. Views (a) and (c) correspond to full colonies, views (b) and (d) to details within this colonies. In (a) and (c) an= ancestrula, in (b) bm= basal male; fz= female zooid; igo= initial growing oocyte; ivo= incipient brooding chamber; and in (d) fz= female zooid. Scale bars are 150 μm in (a) and (c), and 100μm in (b) and (d).

Table 1: Experiment 2, effects of physical contact on basal autozooid area (mm^2) in colonies of C. hyalina. Data are mean ± SD. Values were compared using one-way ANOVA with a Scheffé post hoc test. Values with the same letter are not significantly different ($p>0.05$).

	free-contact side	contact side	control
basal autozooid area (mm^2)	0.101 ± 0.012 a	0.109 ± 0.008 b	0.107 ± 0.010 b

Table 2: Experiment 2, effects of isocontact and allocontact on basal autozooid area (mm^2) in normal and slow growing clones of C. hyalina. Data are mean ± SD. Values were compared using one-way ANOVA with a Scheffé post hoc test. Values with the same letter are not significantly different ($p>0.05$).

	Normal growing clones	Slow growing clones
Isocontact (contact side)	0.096 ± 0.013 a	0.100 ± 0.018 a
Isocontact (contact-free side)	0.104 ± 0.010 b	0.109 ± 0.018 b
Control	0.103 ± 0.009 b	0.110 ± 0.017 b
Allocontact (contact side)	0.102 ± 0.011 a	0.103 ± 0.016 a
Allocontact (contact-free side)	0.110 ± 0.011 b	0.112 ± 0.017 b
Control	0.108 ± 0.009 b	0.109 ± 0.017 ab

Table 3 Experiment 3, effects of thermal shock, desiccation shock and food availability on basal autozooid basal area (mm^2) in colonies of C. hyalina. Values were compared using a simple factorial ANOVA. Data are mean ± SD.

	stressed colonies	control colonies	p
Thermal shock	0.093 ± 0.012	0.110 ± 0.009	<0.0001
Desiccation shock	0.097 ± 0.011	0.108 ± 0.009	<0.0001
Food availability	0.095 ± 0.013	0.106 ± 0.010	<0.0001

Table 4: Experiment 3 summary of effects of mechanical damage on basal autozooid area (mm^2) in colonies of C. hyalina. Data are mean ± SD. In Treatment 1 the mechanical damage was localized in basal autozooids at the growing edge of the colonies and in Treatment 2 in basal autozooids within the colony.

	stressed colonies	Control colonies	p
Treatment 1:	0.100 ± 0.013	0.106 ± 0.011	<0.0001
Treatment 2:	0.101 ± 0.013	0.104 ± 0.012	<0.001

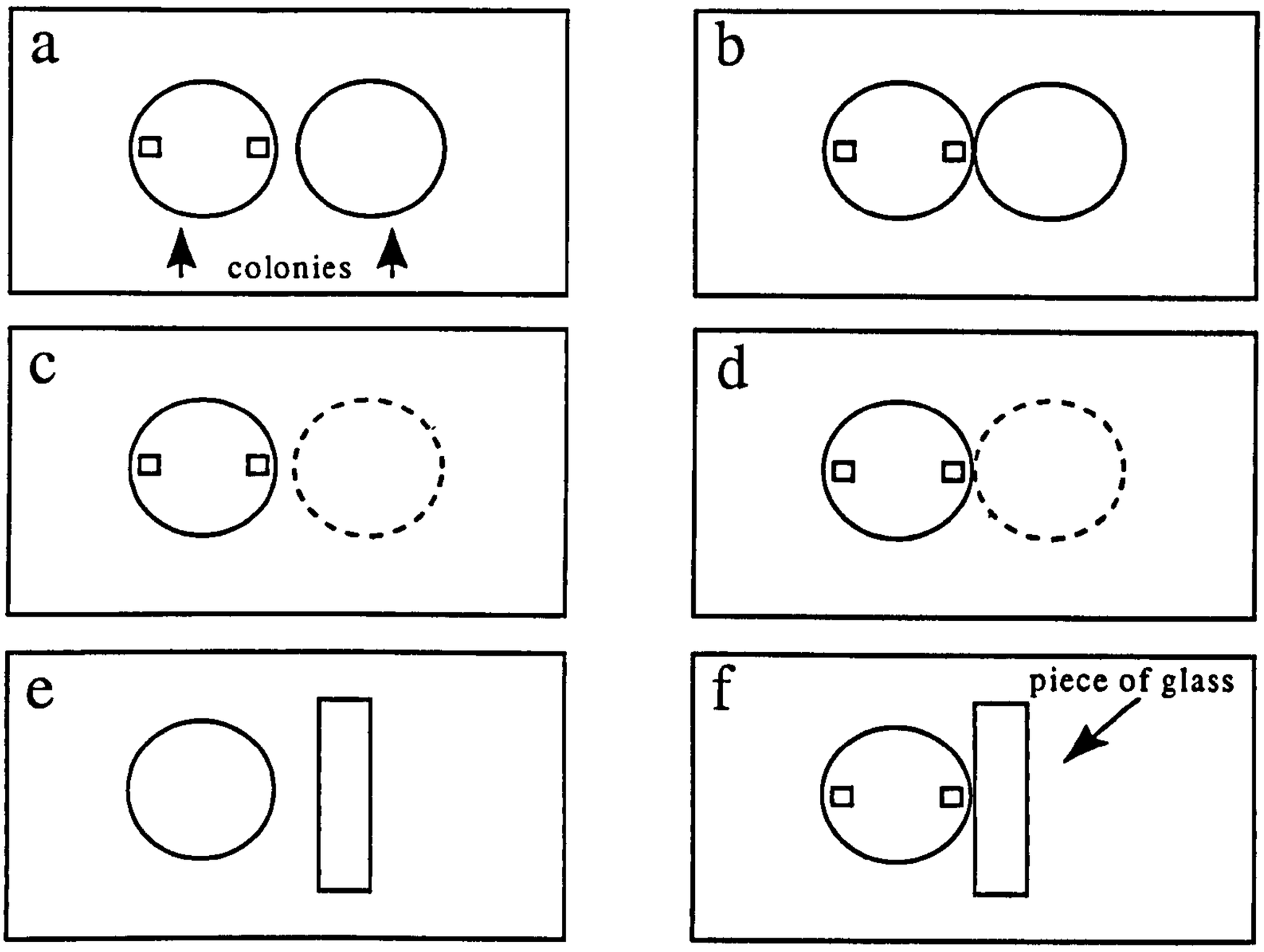

Figure 1: Experiment 2, Diagrammatic representation of the biological and physical contact. Representation (a) and (b) correspond to isocontact before and after colonial contact; (c) and (d) correspond to allocontact before and after contact and, (e) and (f) correspond to artificial contact between colonies and pieces of glass. The small squares within the colonies correspond to areas in the experimental colonies where sampling was carried out.

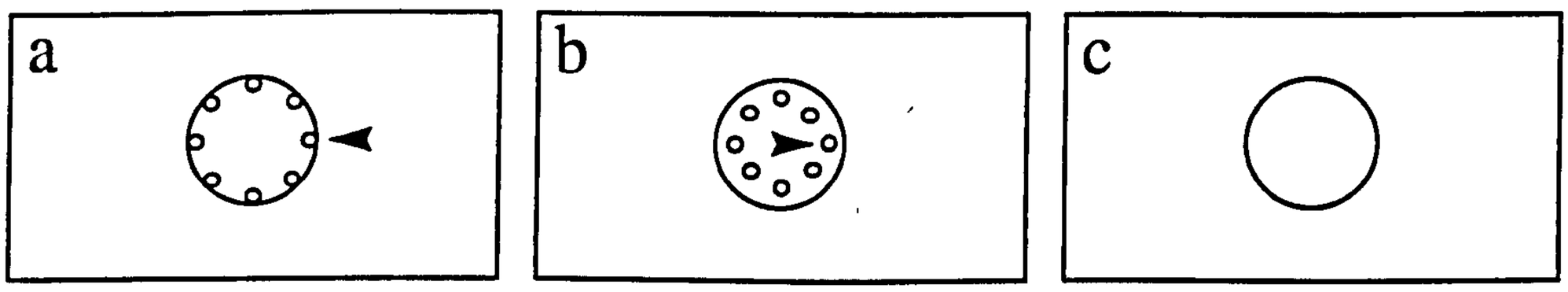

Figure 2: Experiment 3, diagrammatic representation of the mechanical damage in basal zooids at the growing edge of the colonies (a), basal zooids inside the colony (b) and control colonies without damage. The arrows indicate areas in the experimental colonies where basal zooids were damaged.

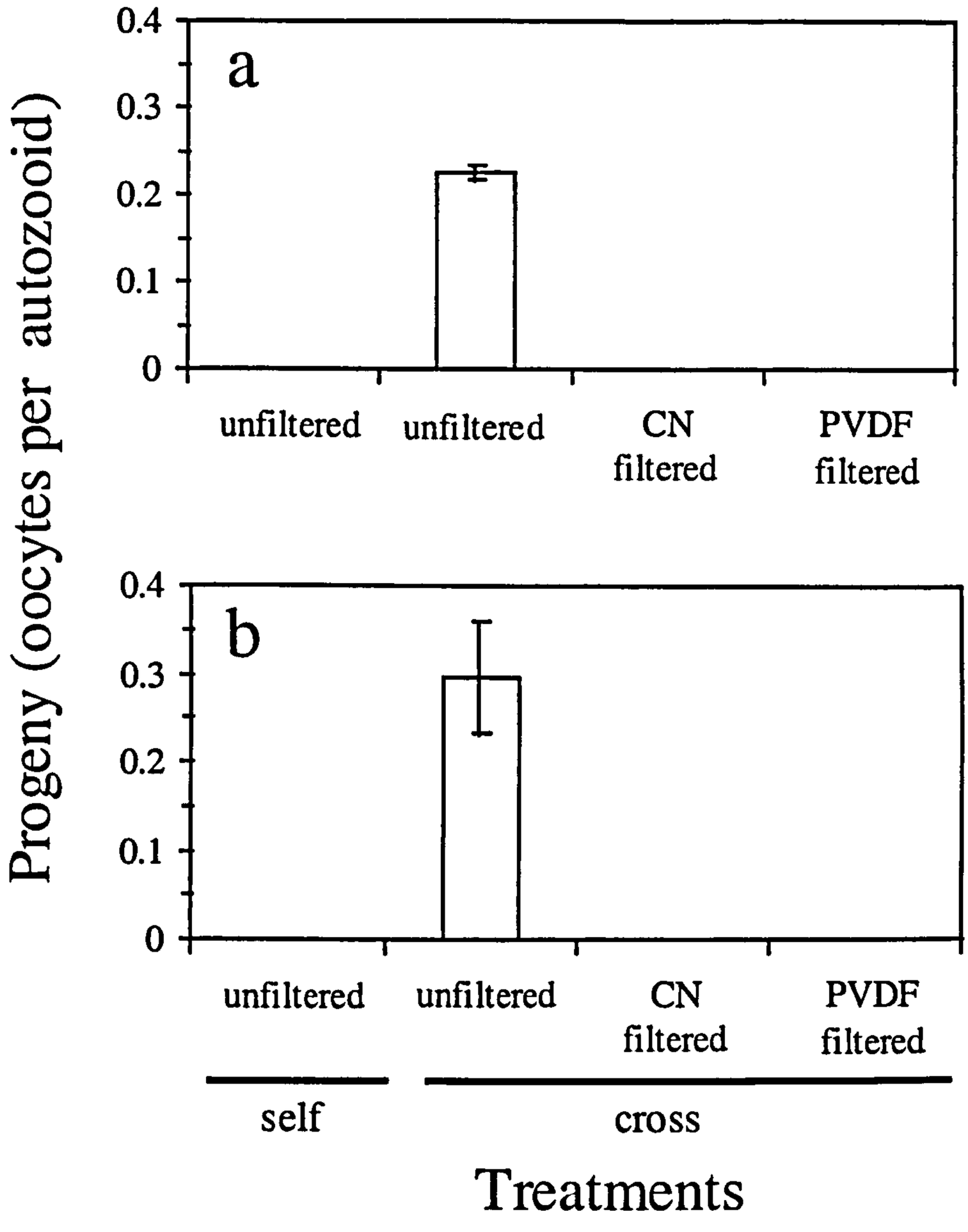

Figure 3: Experiment 1, number of progeny produced by virgin ramets of Celleporella hyalina exposed to water conditioned for 1 h. Colonies were exposed to self unfiltered sperm (Treatment 1), to sexually compatible unfiltered allosperm suspension (Treatment 2), to filtrate of sexually compatible sperm passed through a membrane filter with 0.45 µm pores; cellulose nitrate (Whatman, Midstone, UK) (Treatment 3) and, to filtrate of sexually compatible sperm passed through a membrane filter with 0.45 µm pores; polyninylide difluoride (Millipore, Watford, UK) (Treatment 4). This experiment was divided in two parts. In the first part, ramets from clone J_1 were used as allosperm donor and ramets of clone Q_1 used as receptor colonies (a), in the second part, ramets from clone A_1 were used as allosperm donor and ramets of clone M_1 used as receptor colonies (b). Values are mean ± SE

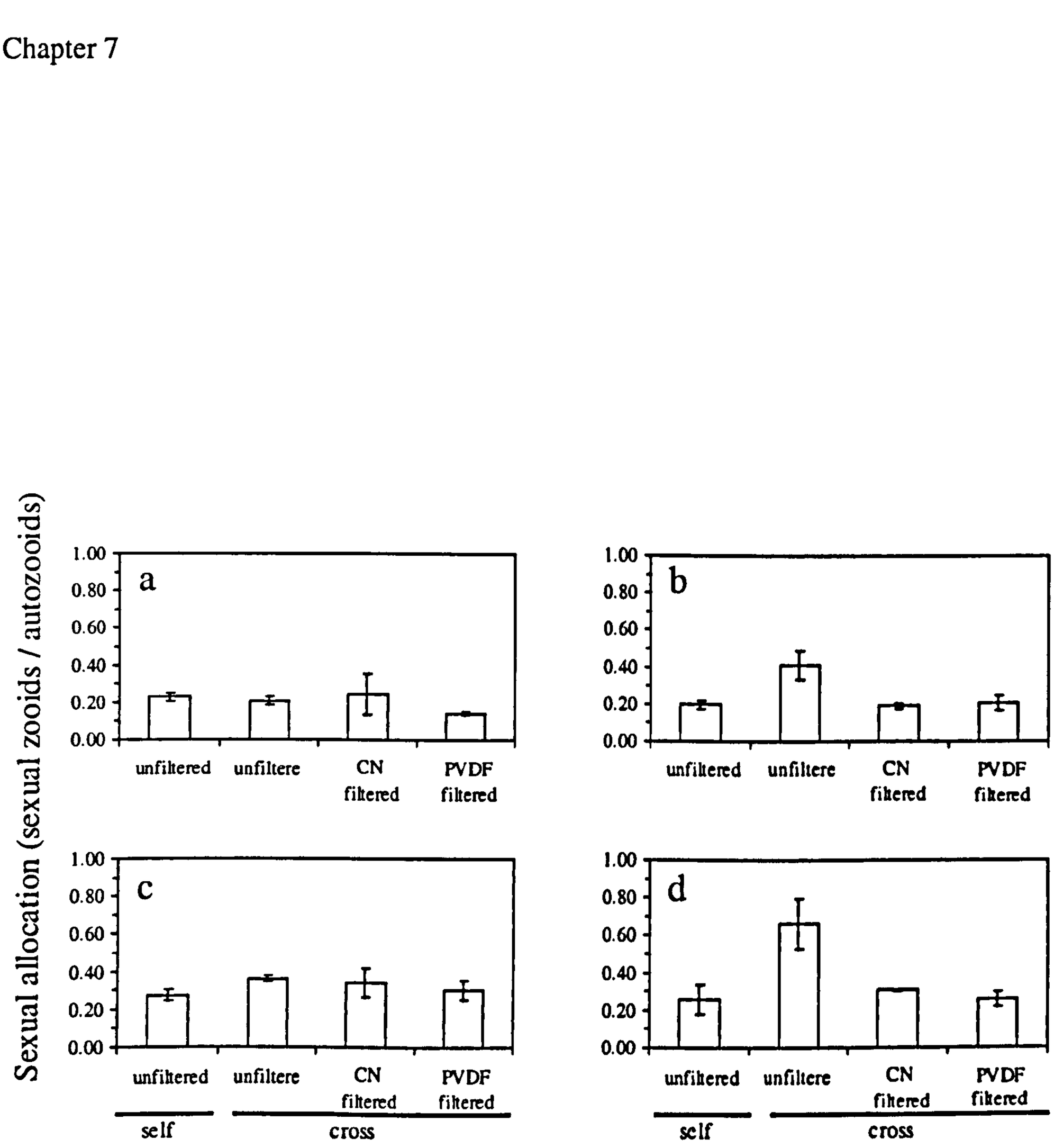

Figure 4: Experiment 1, sexual zooids per active basal zooid (sexual allocation) in part 1 (top graphs) and part 2 (bottom graphs) of this experiment. Graphs on the left correspond to values before exposure to water conditioned for 1 h, and graphs on the right to values after 8 wk. See Figure 1 for a complete legend. Values are mean ± SD.

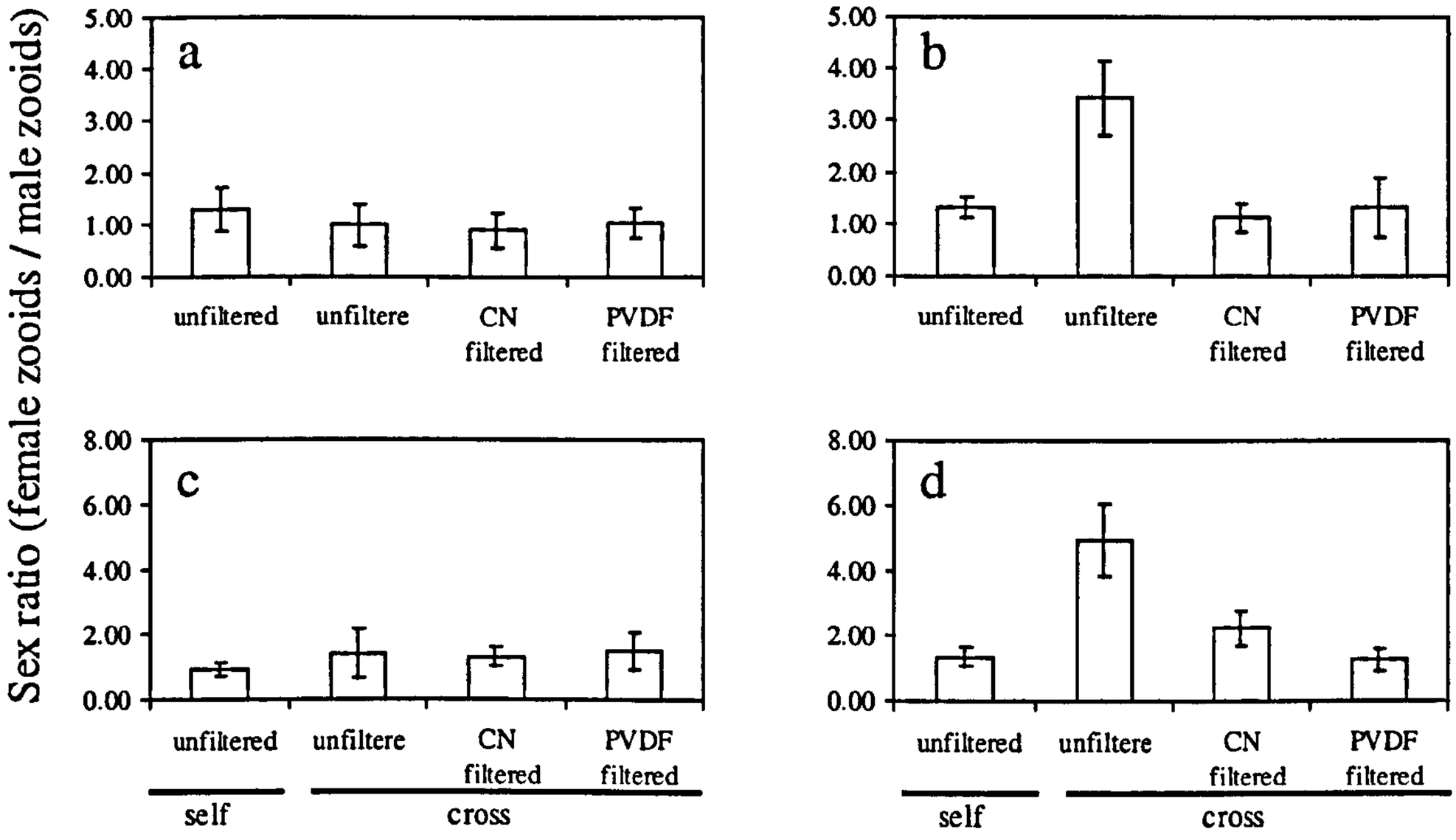

Figure 5: Experiment 1, number of sex ratio (female zooids / male zooids) in part 1 (top graphs) and part 2 (bottom graphs) of this experiment. See Figure 2 for a complete legend. Values are mean ± SD.

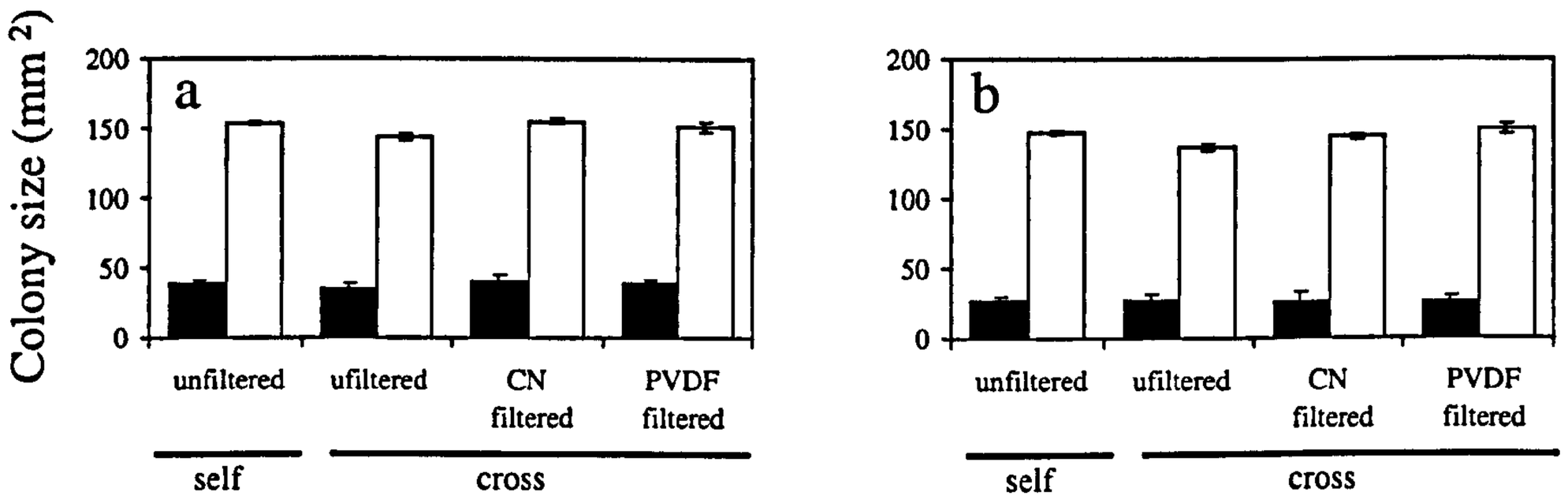

Figure 6: Experiment 1, colony size in virgin ramets of Celleporella hyalina exposed to water conditioned for 1 h. Black bars correspond to the colony size at the beginning of the experiments one day before the colonies were exposed to water conditioned for 1 h. White bars correspond to the colony size 8 wk later. See Figure 3 for a complete legend. Values are mean ± SD.

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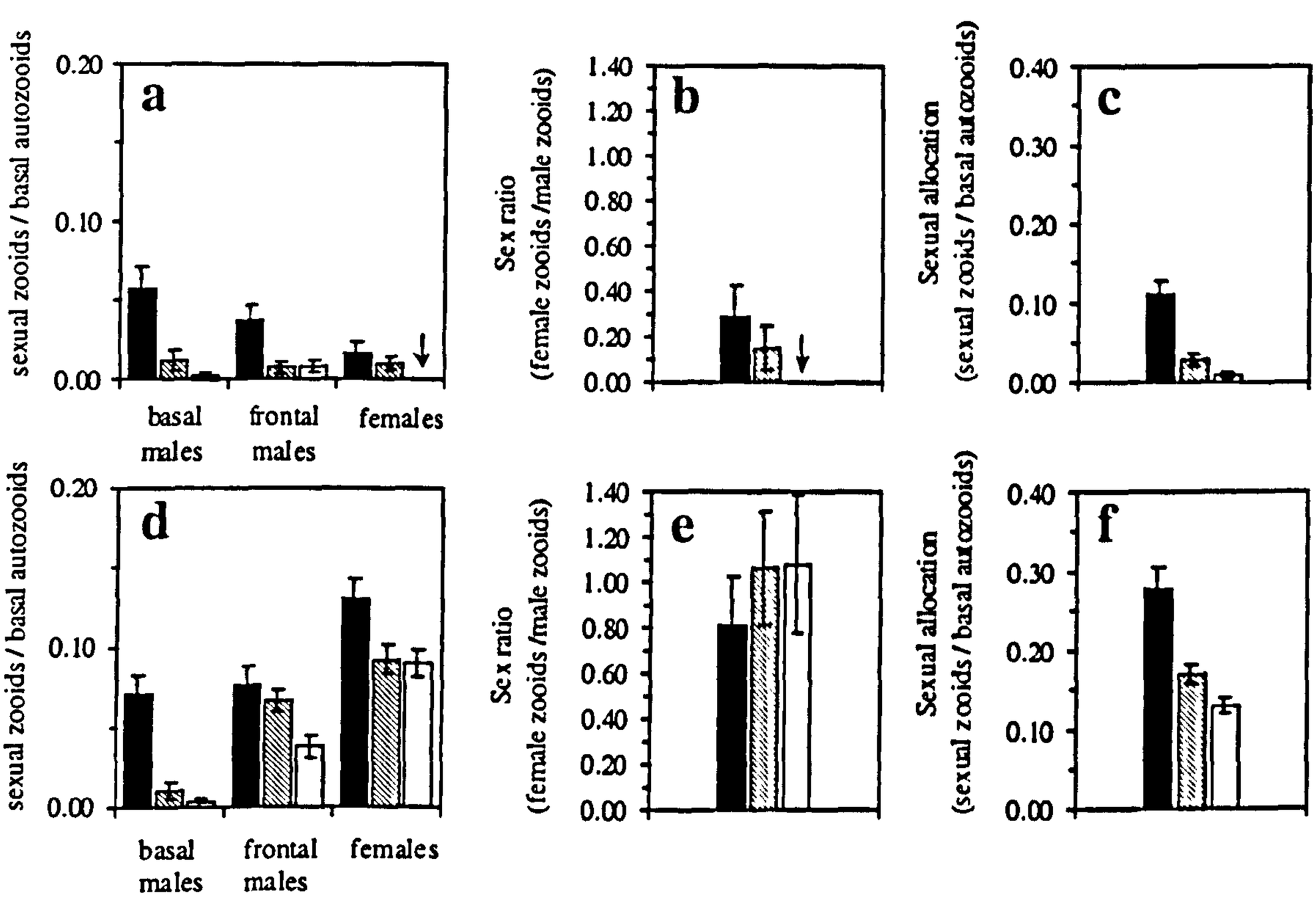


Figure 7: Experiment 2, proportion of sexual zooids, sex ratio and sexual investment in contact between normally growing (graphs a, b and c) and slow growing genotypes (graphs d, e and f) of C. hyalina and a piece of glass. Black bars correspond to samples in colonies in the contact area, shaded bars correspond to samples on the opposite side, and white bars to control colonies without contact. Data are mean ± SE, and the arrows indicate zeros.

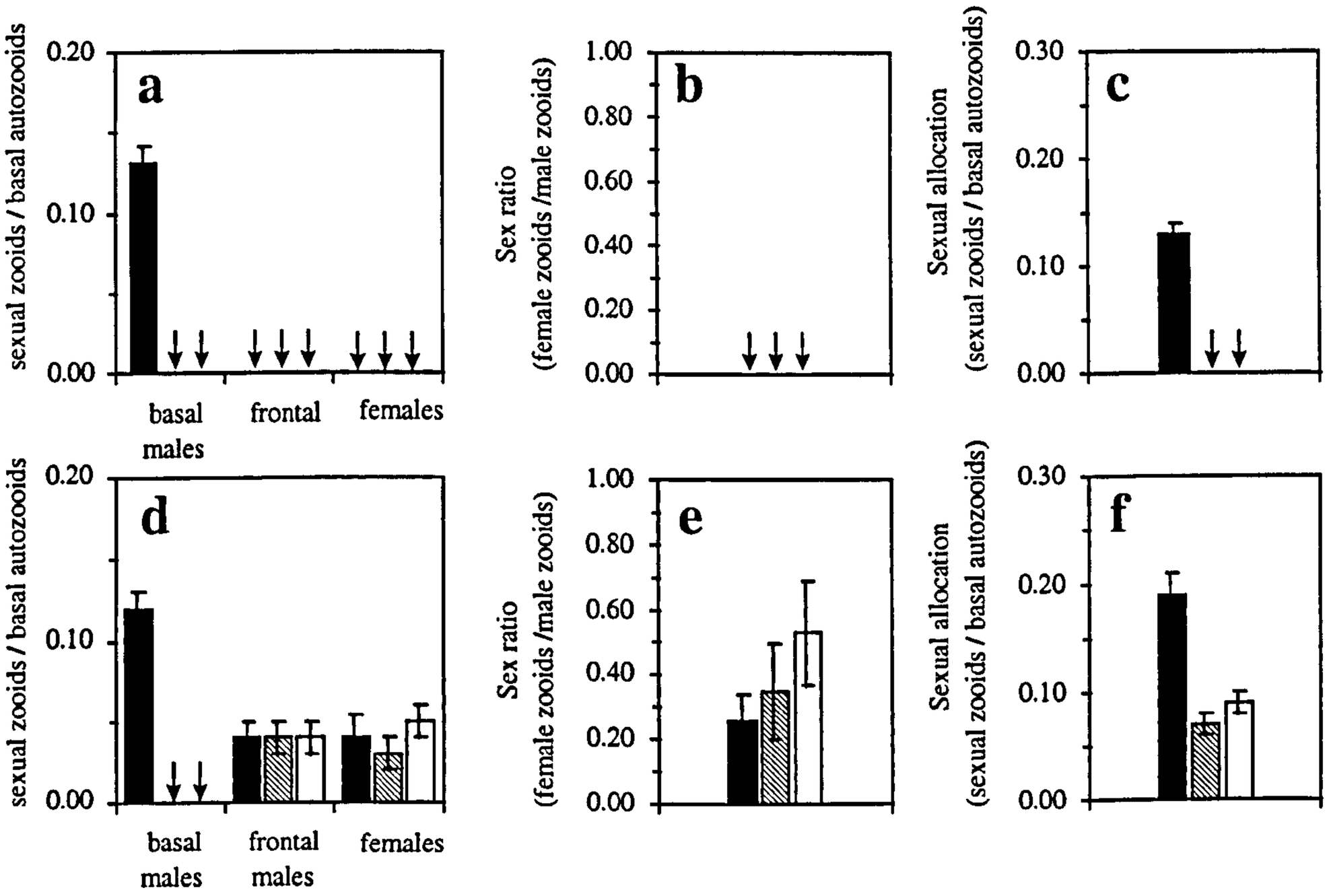


Figure 8: Experiment 2, proportion of sexual zooids, sex ratio and sexual allocation in isocontact experiments between normally growing (graphs a, b and c) and slow growing genotypes (graphs d, e and f) of C. hyalina. Black bars correspond to samples in colonies in the contact area, shaded bars correspond to samples on the opposite side, and white bars to control colonies without contact. Data are mean ± SE, and the arrows indicate zeros.

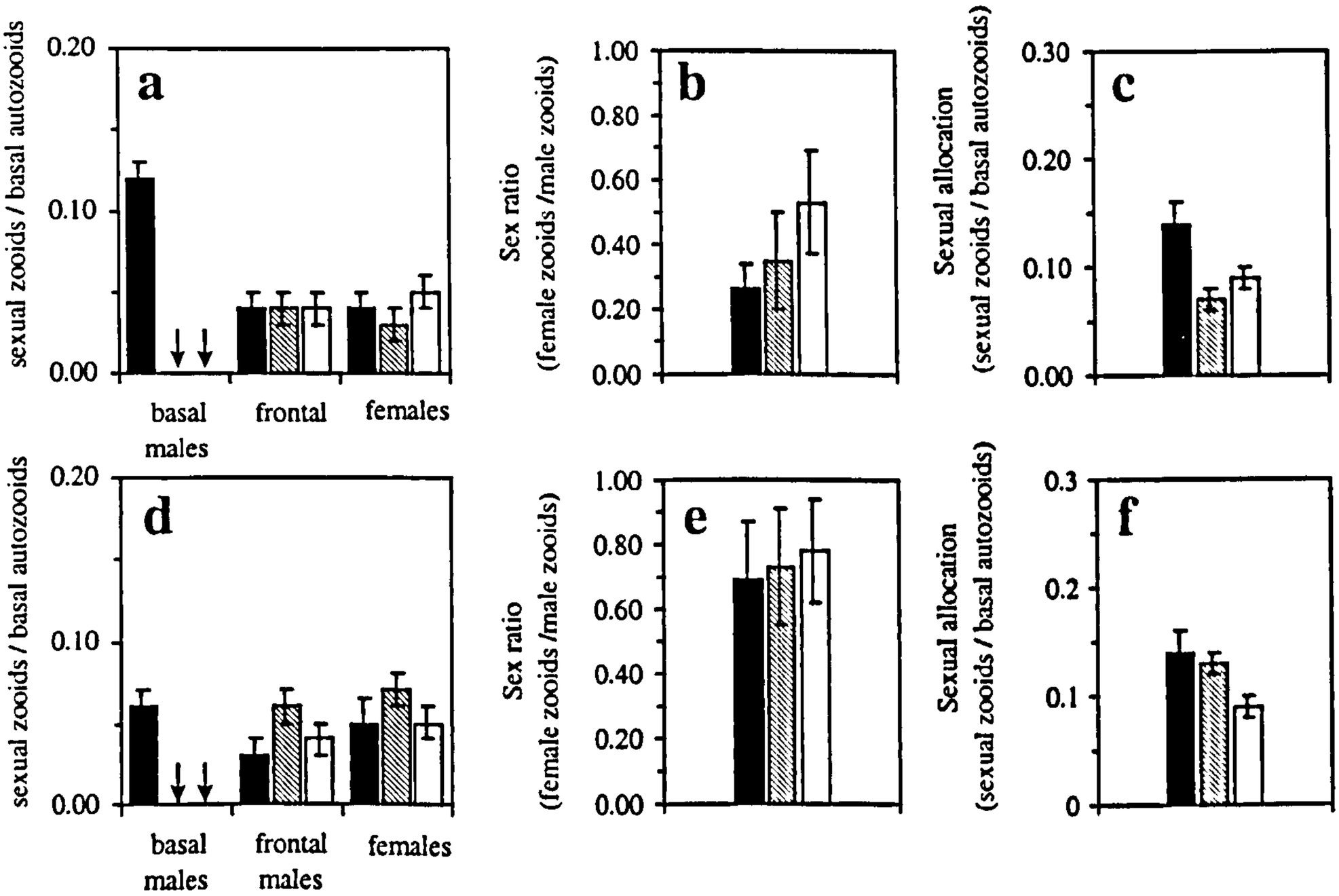


Figure 9: Experiment 2, proportion of sexual zooids, sex ratio and sexual allocation in allocontacts between normally growing (graphs a, b and c) and slow growing genotypes (graphs d, e and f) of C. hyalina. Black bars correspond to samples in colonies in the contact area, shaded bars correspond to samples on the opposite side, and white bars to control colonies without contact. Data are mean ± SE, and the arrows indicate zeros.

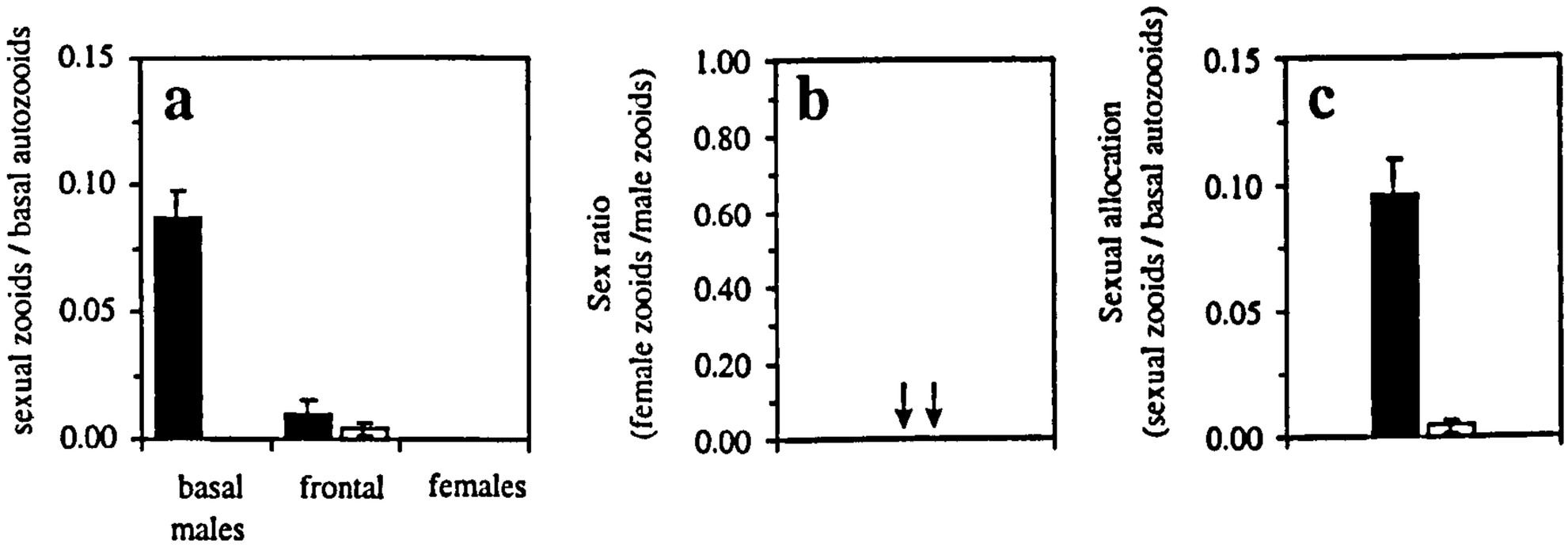


Figure 10: Experiment 3, proportion of sexual zooids to basal autozooids, sex ratio and sexual allocation in colonies exposed to thermal stress (black bars) and control colonies (white bars). Data are mean ± SE, and the arrows indicate zeros.

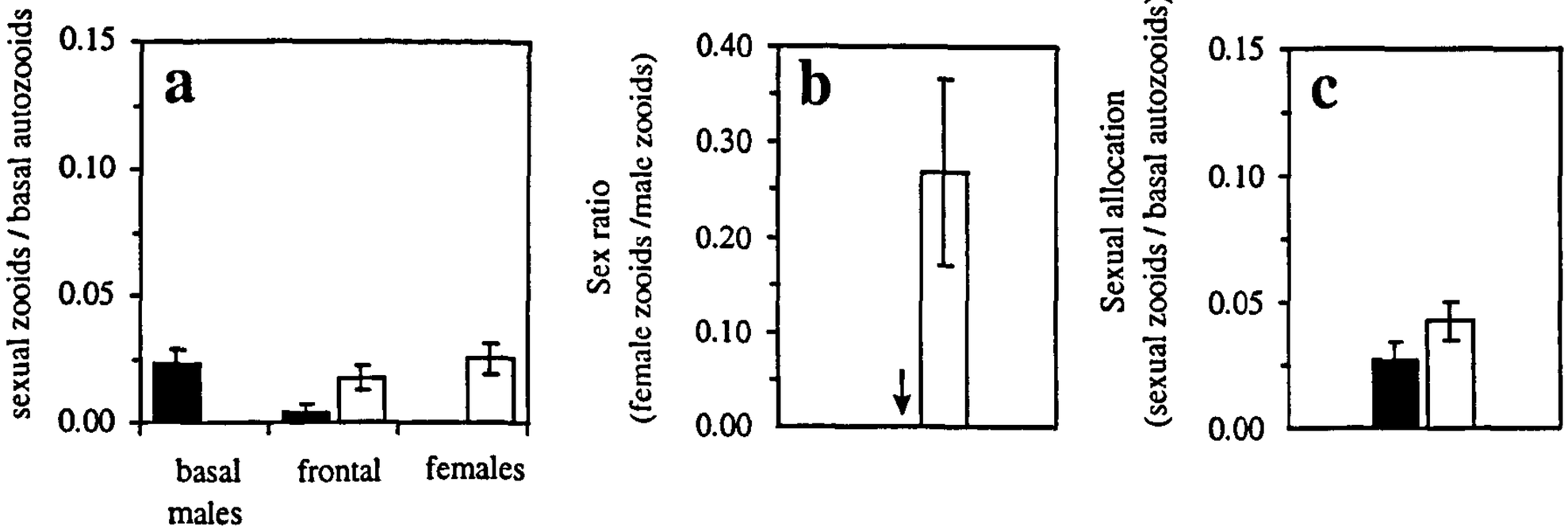


Figure 11: Experiment 3, proportion of sexual zooids to basal autozooids, sex ratio and sexual allocation in colonies exposed to desiccation stress (black bars) and control colonies (white bars). Data are mean ± SE, and the arrows indicate zeros.

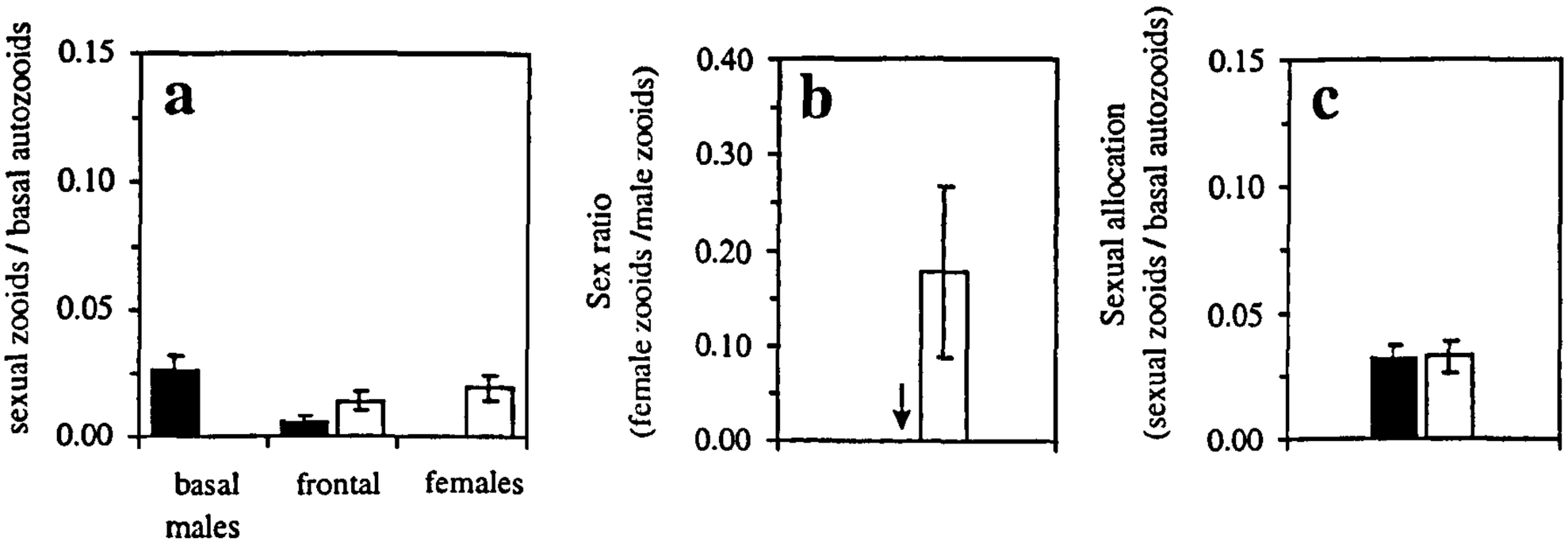


Figure 12: Experiment 3, proportion of sexual zooids to basal autozooids, sex ratio and sexual investment in colonies exposed to food availability stress (black bars) and control colonies (white bars). Data are mean ± SE, and the arrows indicate zeros.

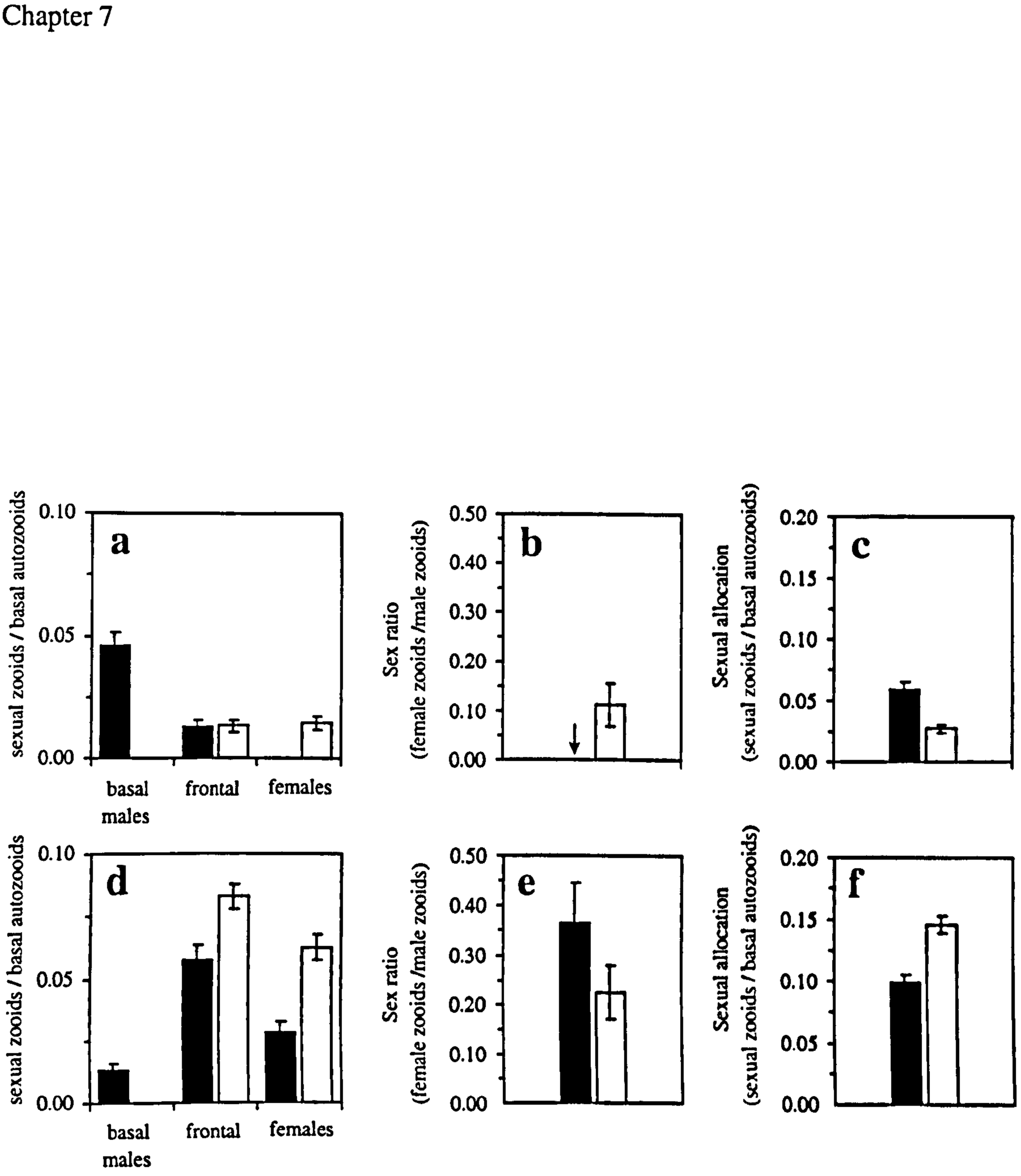
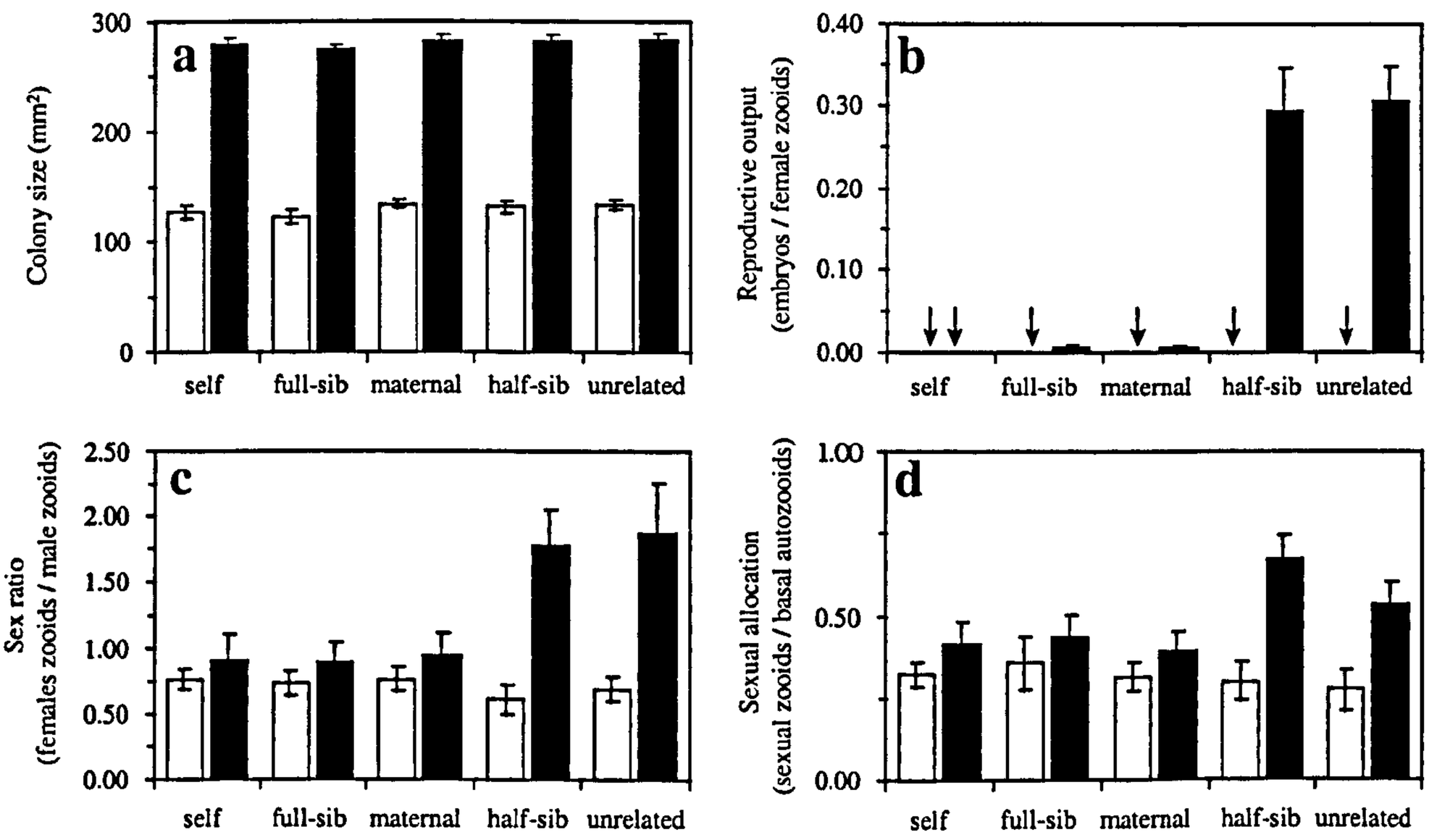


Figure 13: Experiment 3, proportion of sexual zooids to basal autozooids, sex ratio and sexual allocation in colonies exposed to mechanical stress at the original growing edge (a, b and c) and apart from the original growing edge of the colonies (d, e and f). Black bars correspond to stressed colonies and white bars to control colonies. Data are mean ± SE, and the arrows indicate zeros.



Relatedness between sperm suspension and sperm receptors colonies

Figure 14: Experiment 4, colony size, reproductive output, sex ratio and sexual allocation in colonies of 6 different genotypes exposed to sperm suspensions of different genetic relatedness. White bars correspond to values at the beginning of the experiment and the black bars to 2 mo later. Data are mean ± SE, and the arrows indicate zeros.

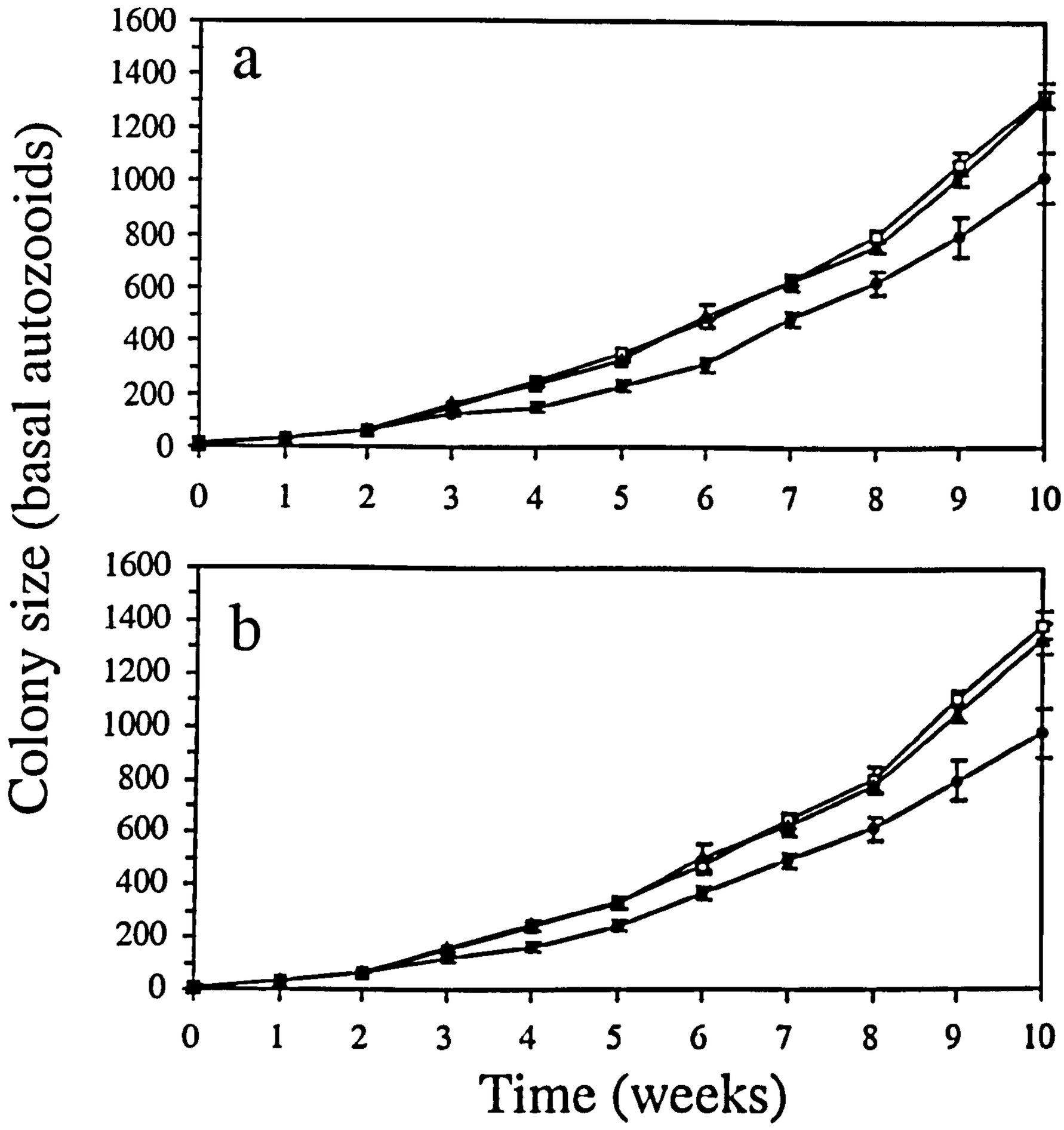


Figure 15: Experiment 5, Growth in colonies of C. hyalina under three different regimes of sperm dosage (● represent colonies exposed daily to allosperm suspensions; represent control colonies maintained in reproductive isolation and ▲ represent colonies exposed to allosperm suspensions on the first day). The top graph (a) corresponds to part 1 and the bottom graph (b) to part 2. All the colonies were 10 active basal autozooids at the beginning of the experiment. Data are mean number of autozooids (± SE).

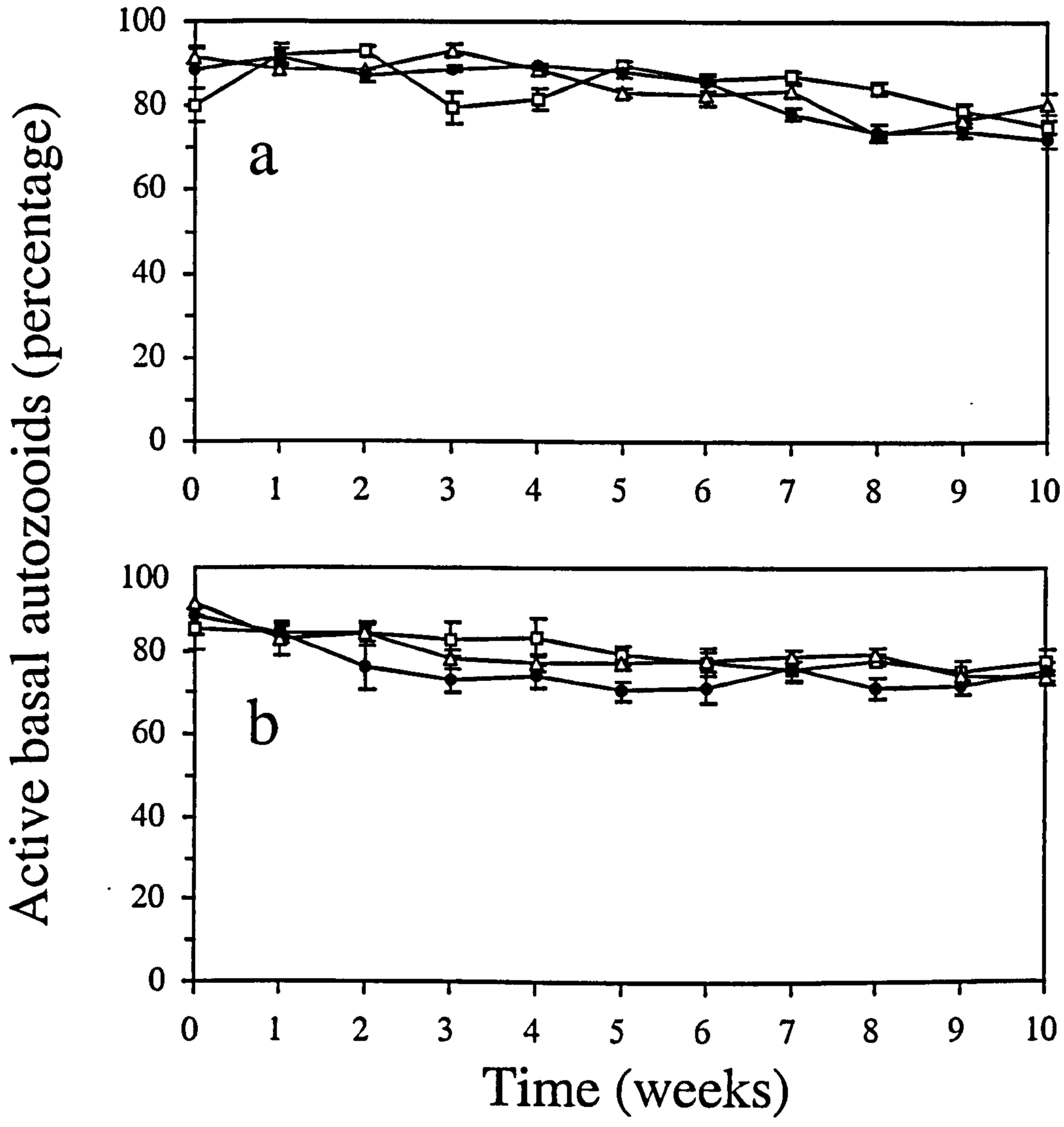


Figure 16: Experiment 5, Percentage of active basal zooids in colonies of C. hyalina under three different regimes of sperm dosage. Data are mean ± SE. See Figure 15 for the complete legend.

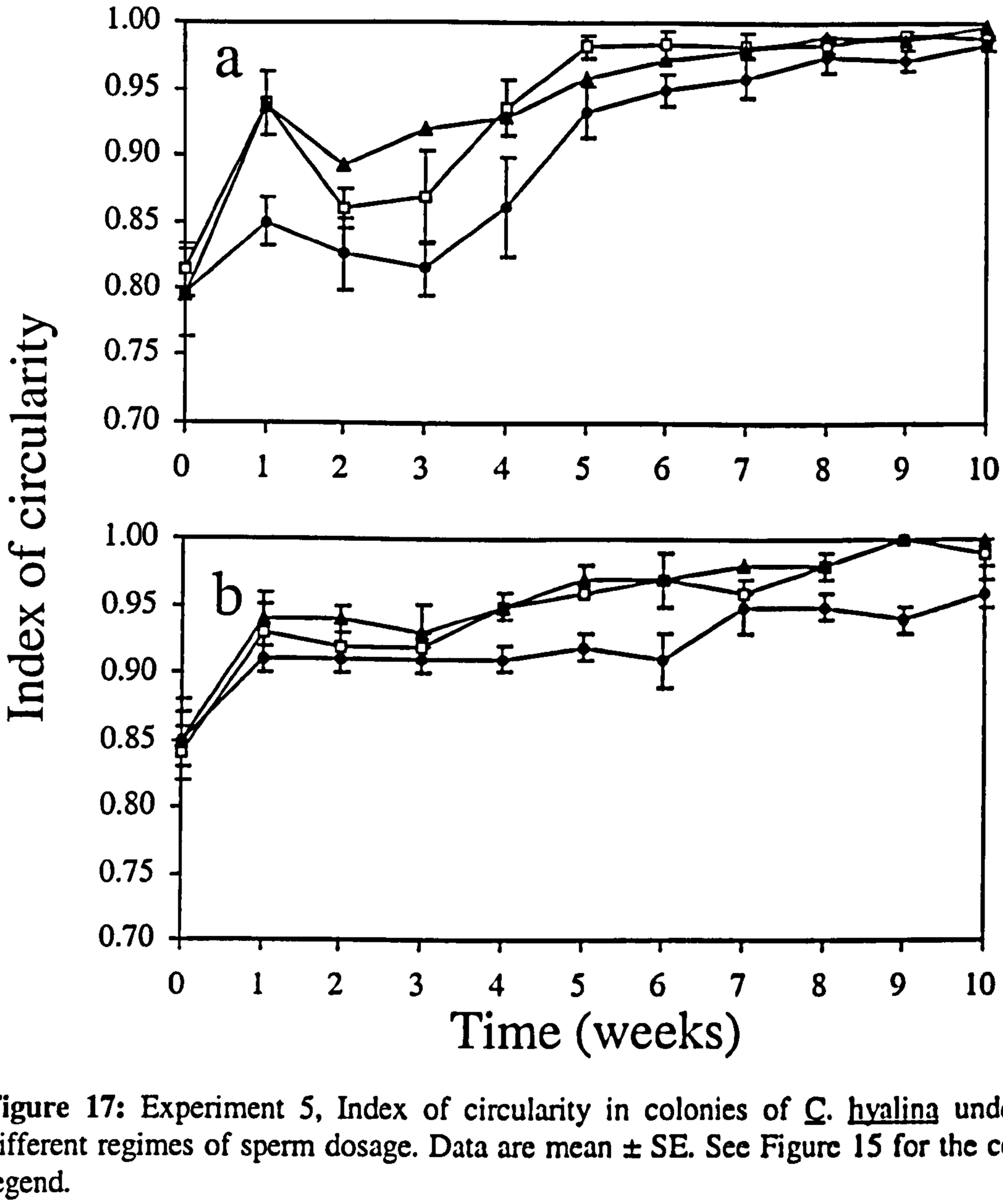


Figure 17: Experiment 5, Index of circularity in colonies of C. hyalina under three different regimes of sperm dosage. Data are mean ± SE. See Figure 15 for the complete legend.

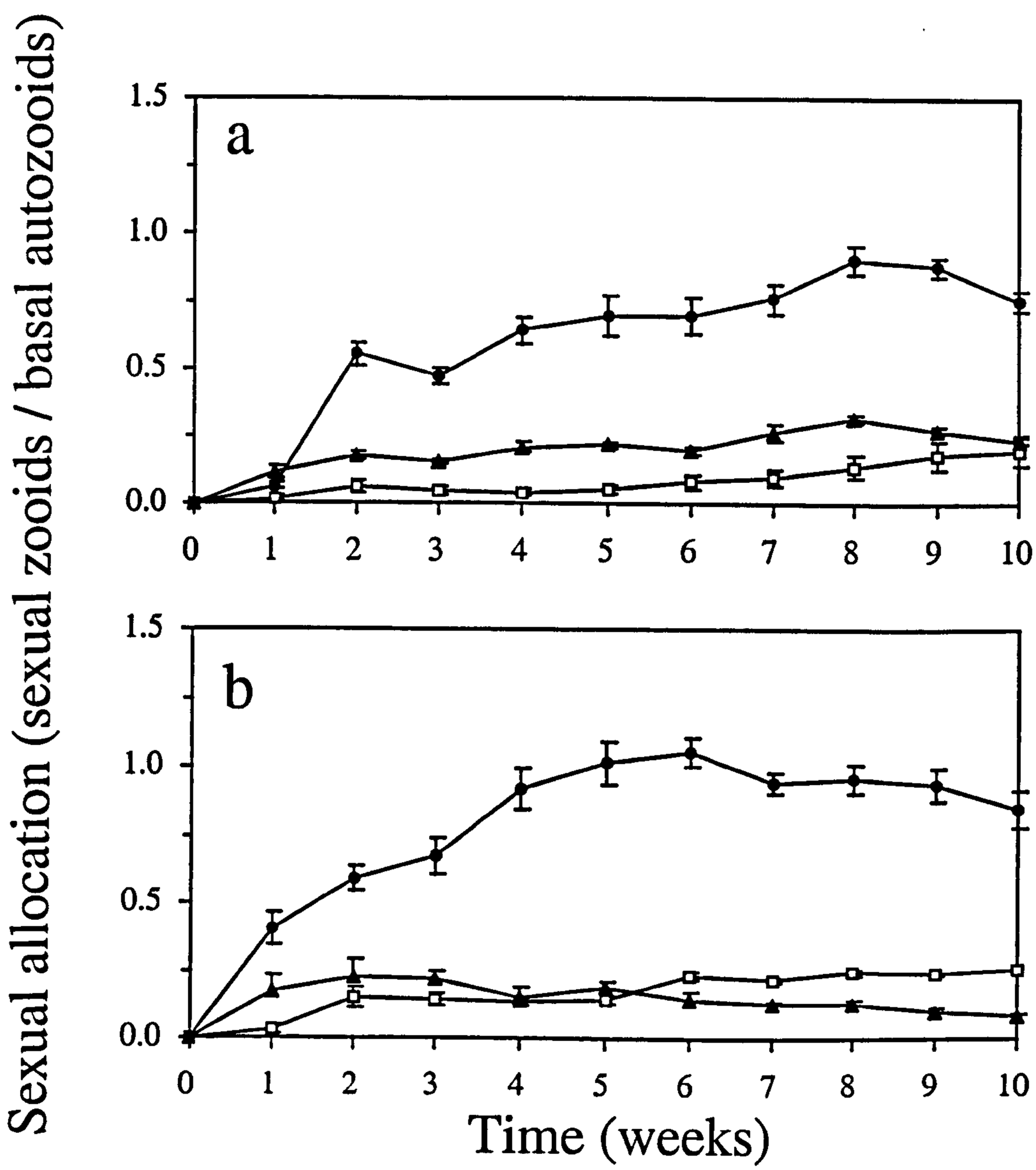


Figure 18: Experiment 5, Sexual allocation in colonies of C. hyalina under three different regimes of sperm dosage. Data are mean ± SE. See Figure 15 for the complete legend.

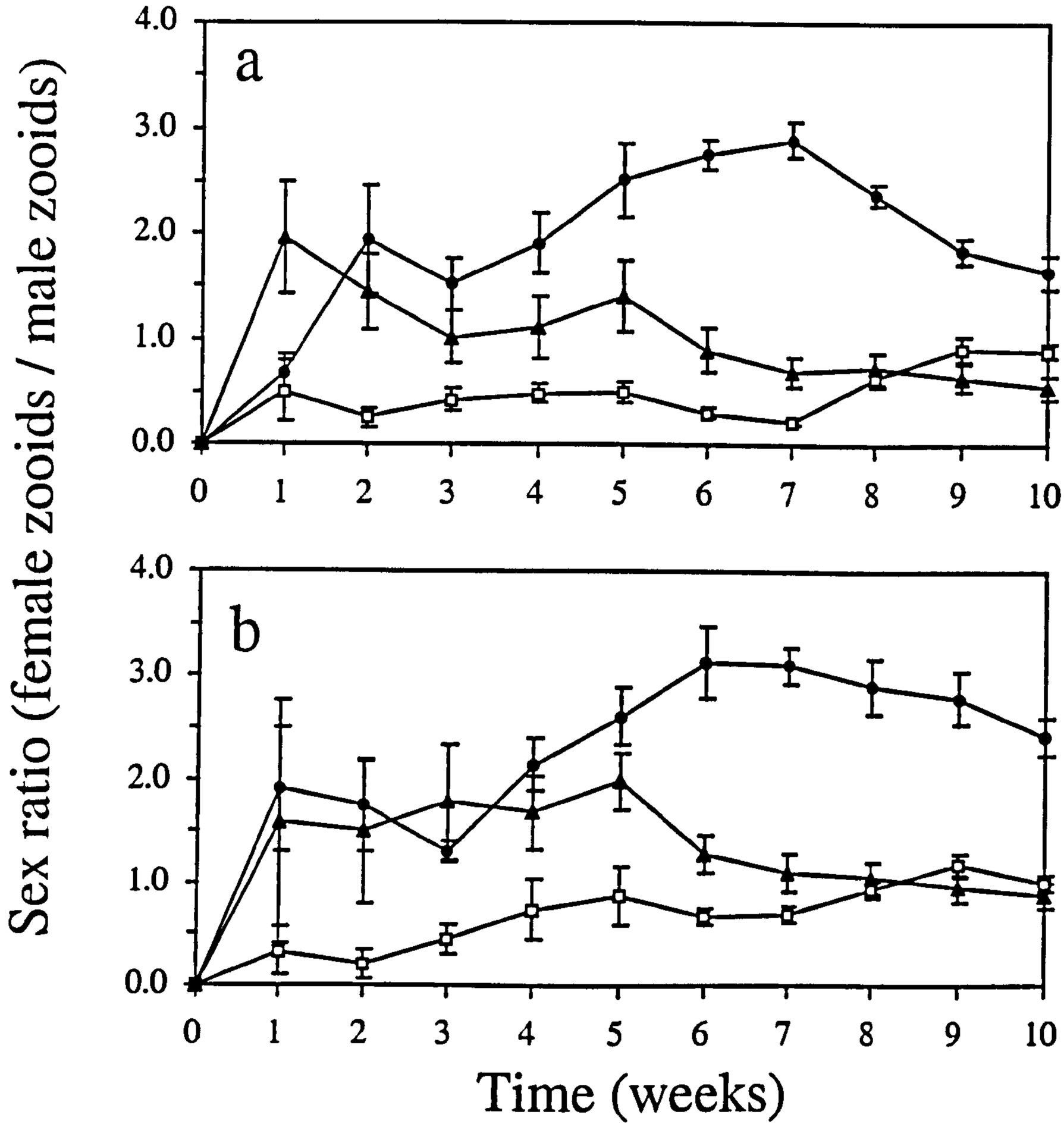


Figure 19: Experiment 5, Sex ratio in colonies of C. hyalina under three different regime of sperm dosage. Data are mean ± SE. See Figure 7 for the complete legend.

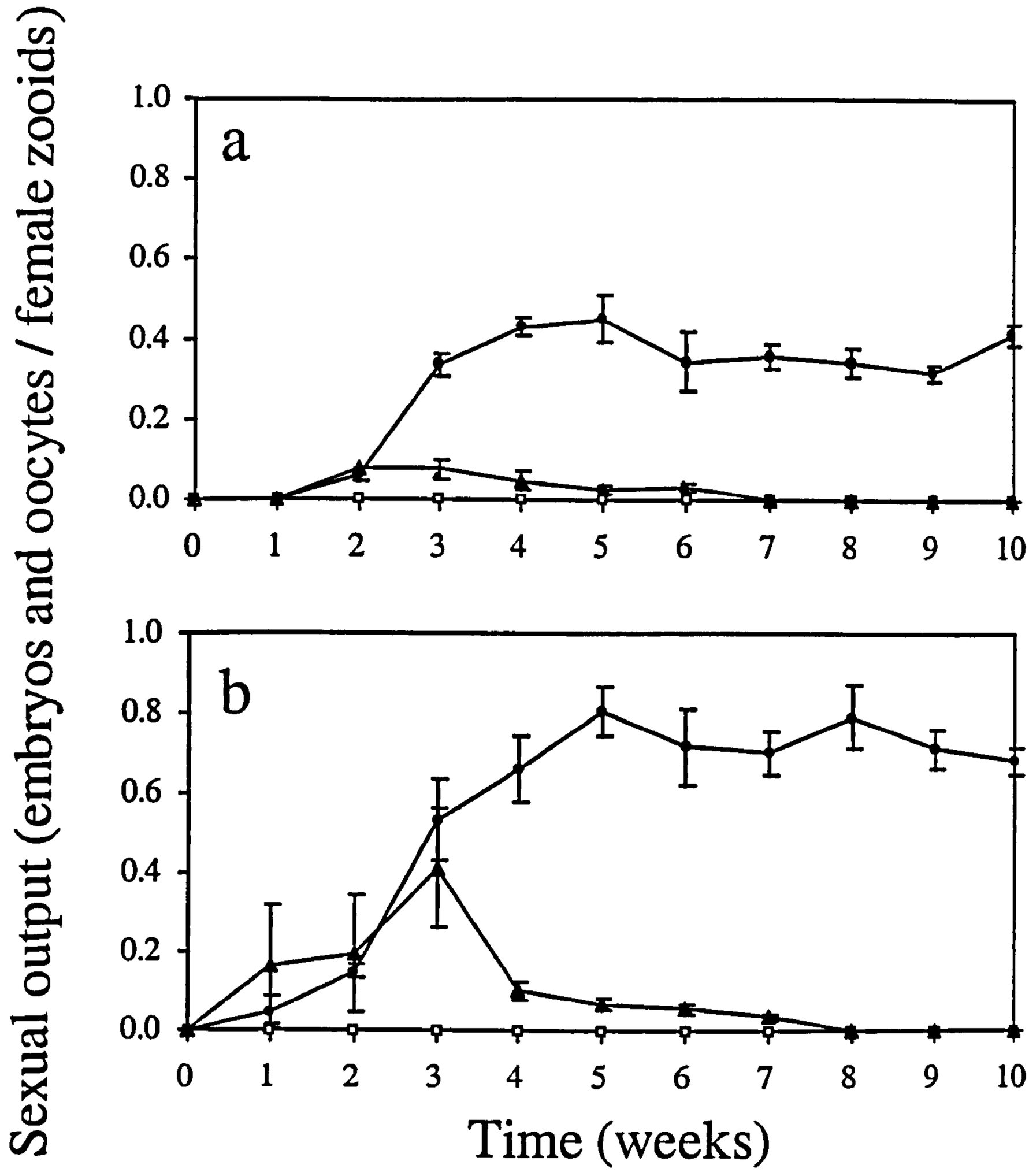


Figure 20: Experiment 5, Sexual output in colonies of C. hyalina under three different regimes of sperm dosage. Data are mean ± SE. See Figure 15 for the complete legend.

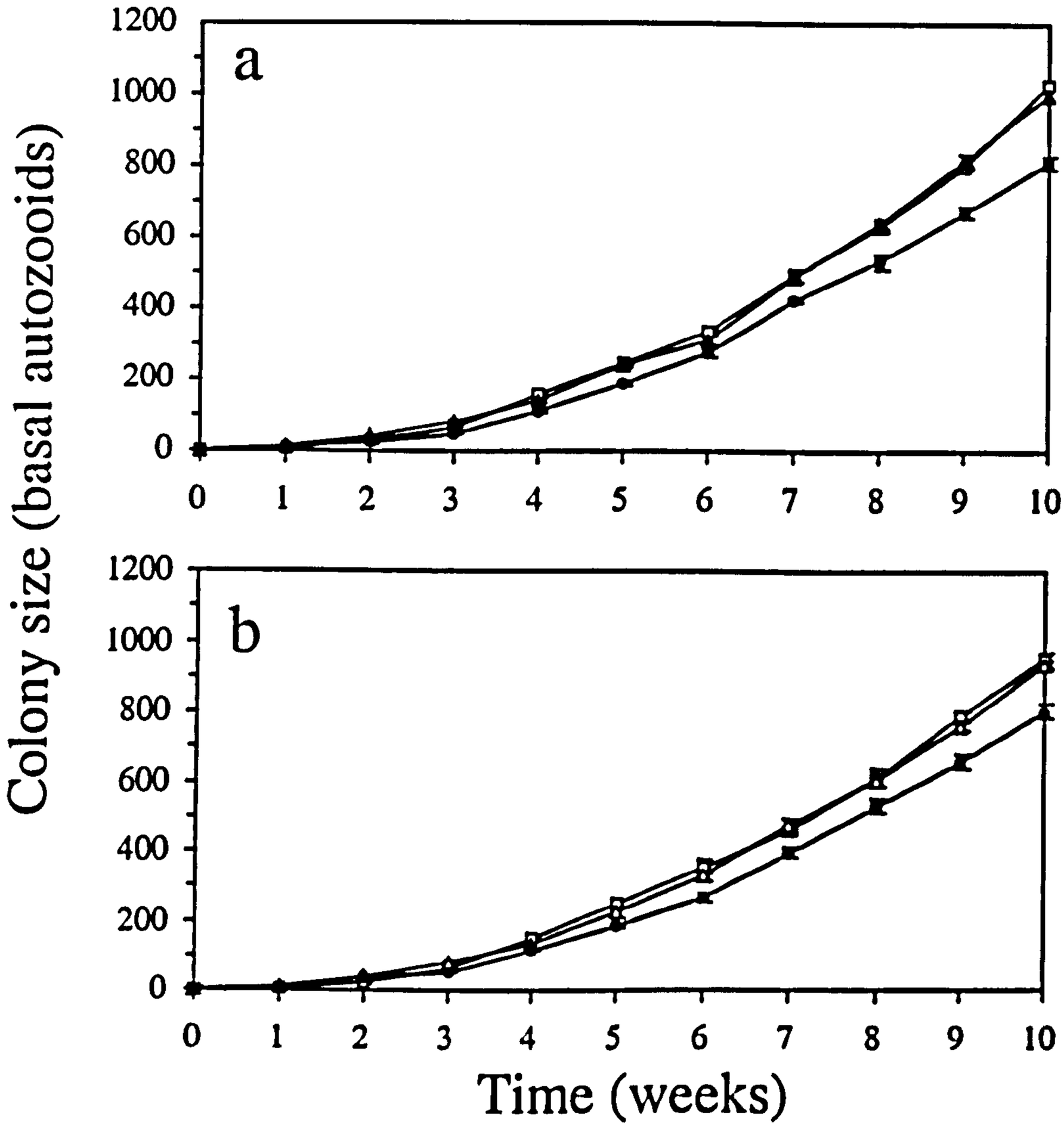


Figure 21: Experiment 5, Growth in colonies of C. hyalina under three different regimes of sperm dosage (● represent colonies exposed daily to allosperm suspensions; represent control colonies maintained in reproductive isolation and ▲ represent colonies exposed to allosperm suspensions on the first day). The top graph (a) corresponds to the part 1 and the bottom graph (b) to the part 2. All the colonies were ancestrulae plus two basal active autozooids at the beginning of the experiment. Data are mean ± SE.

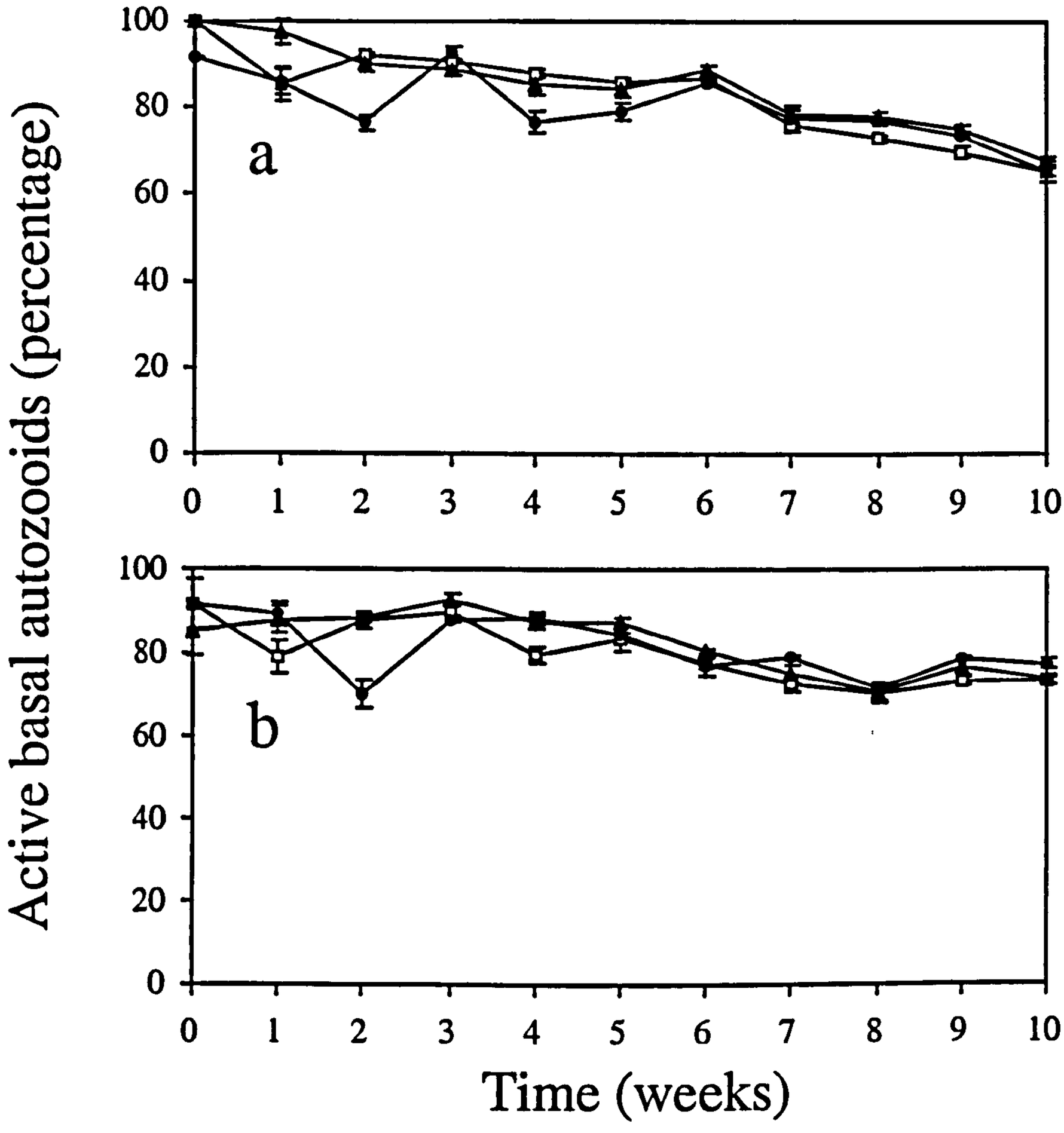


Figure 22: Experiment 5, Percentage of active basal zooids in colonies of C. hyalina under three different regimes of sperm dosage. Data are mean ± SE. See figure 21 for the complete legend.

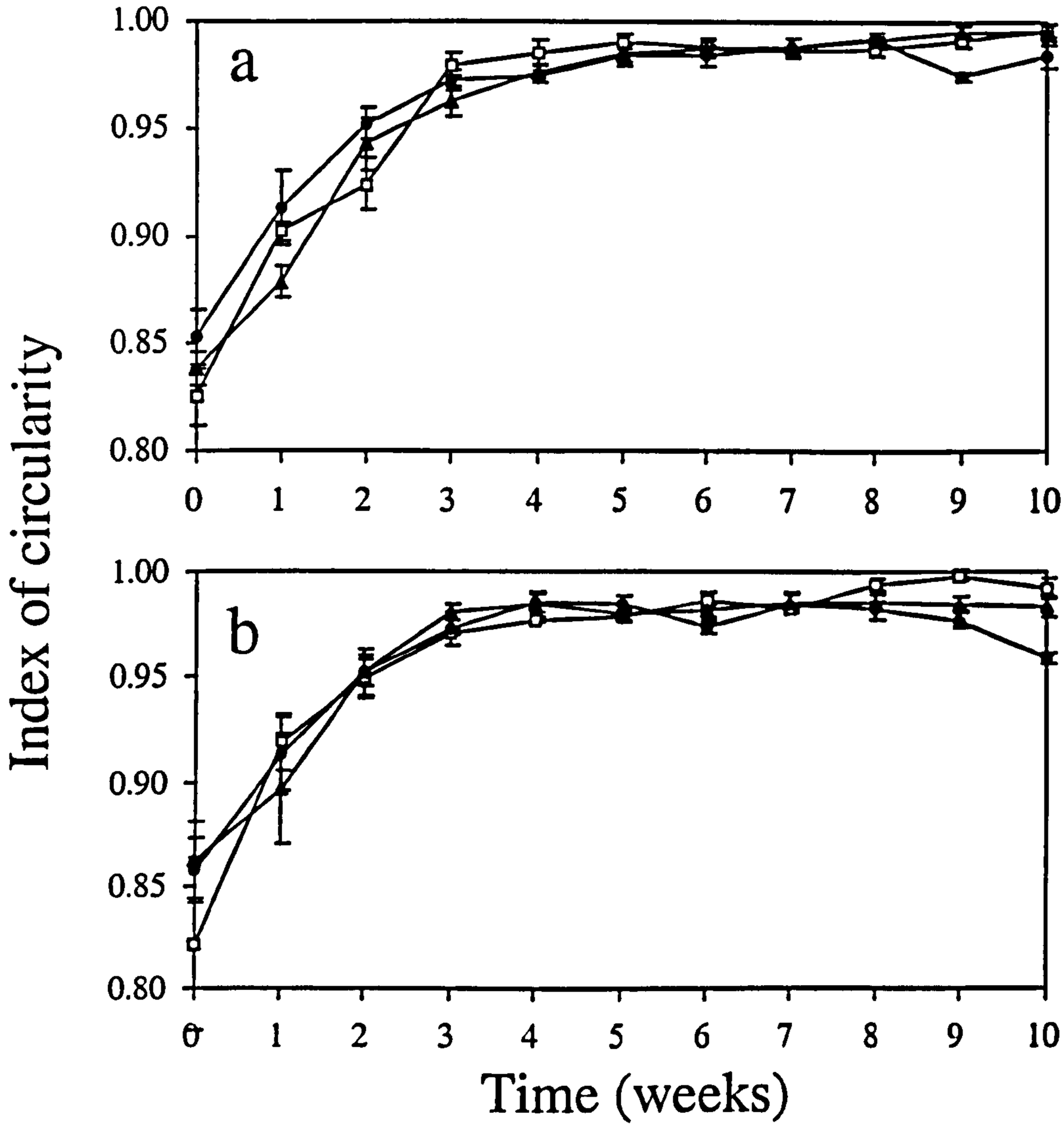


Figure 23: Experiment 5, Index of circularity in colonies of C. hyalina under three different regimes of sperm dosage. Data are mean ± SE. See Figure 21 for the complete legend.

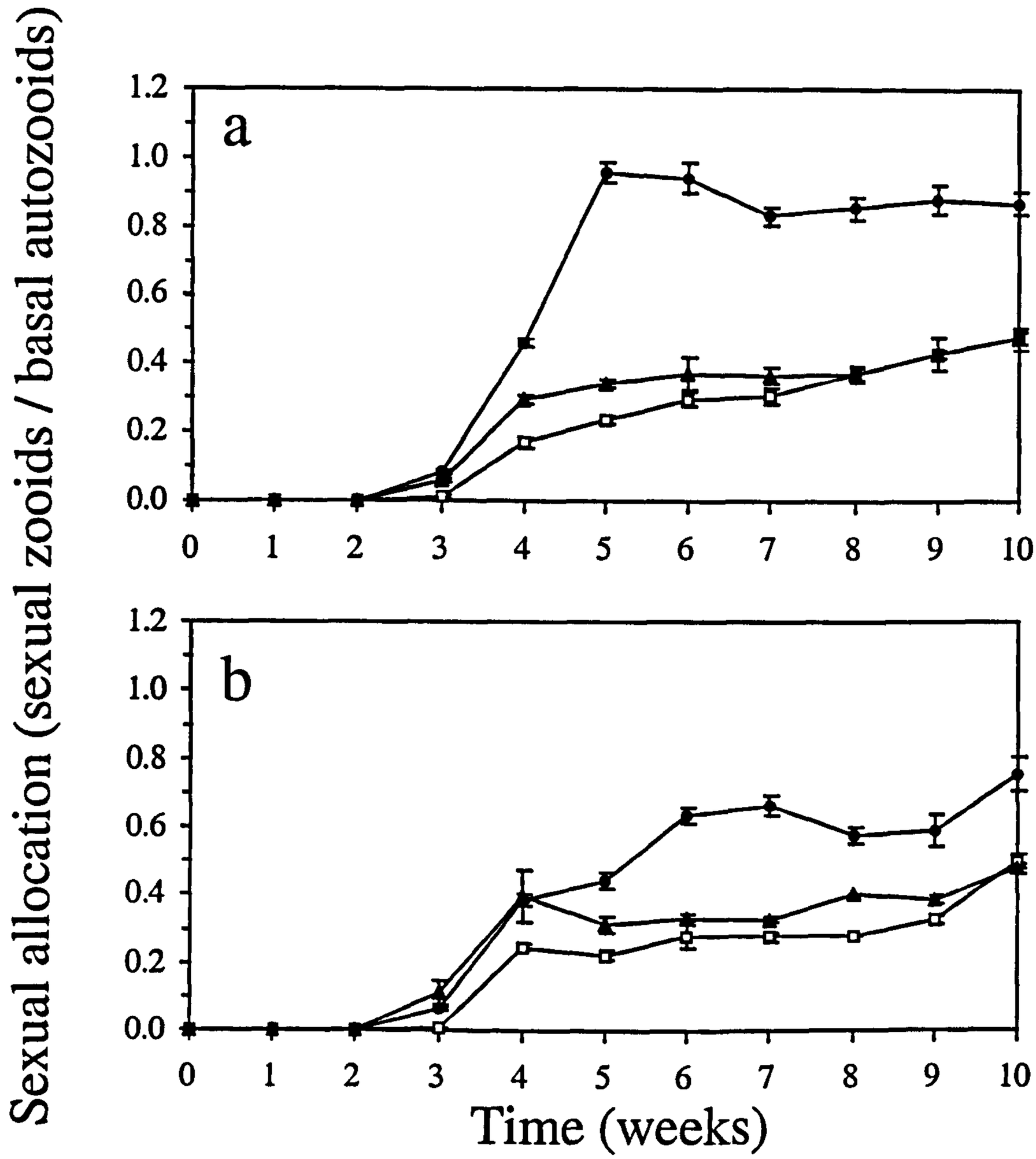


Figure 24: Experiment 5, Sexual allocation in colonies of C. hyalina under three different regimes of sperm dosage. Data are mean ± SE. See Figure 21 for the complete legend.

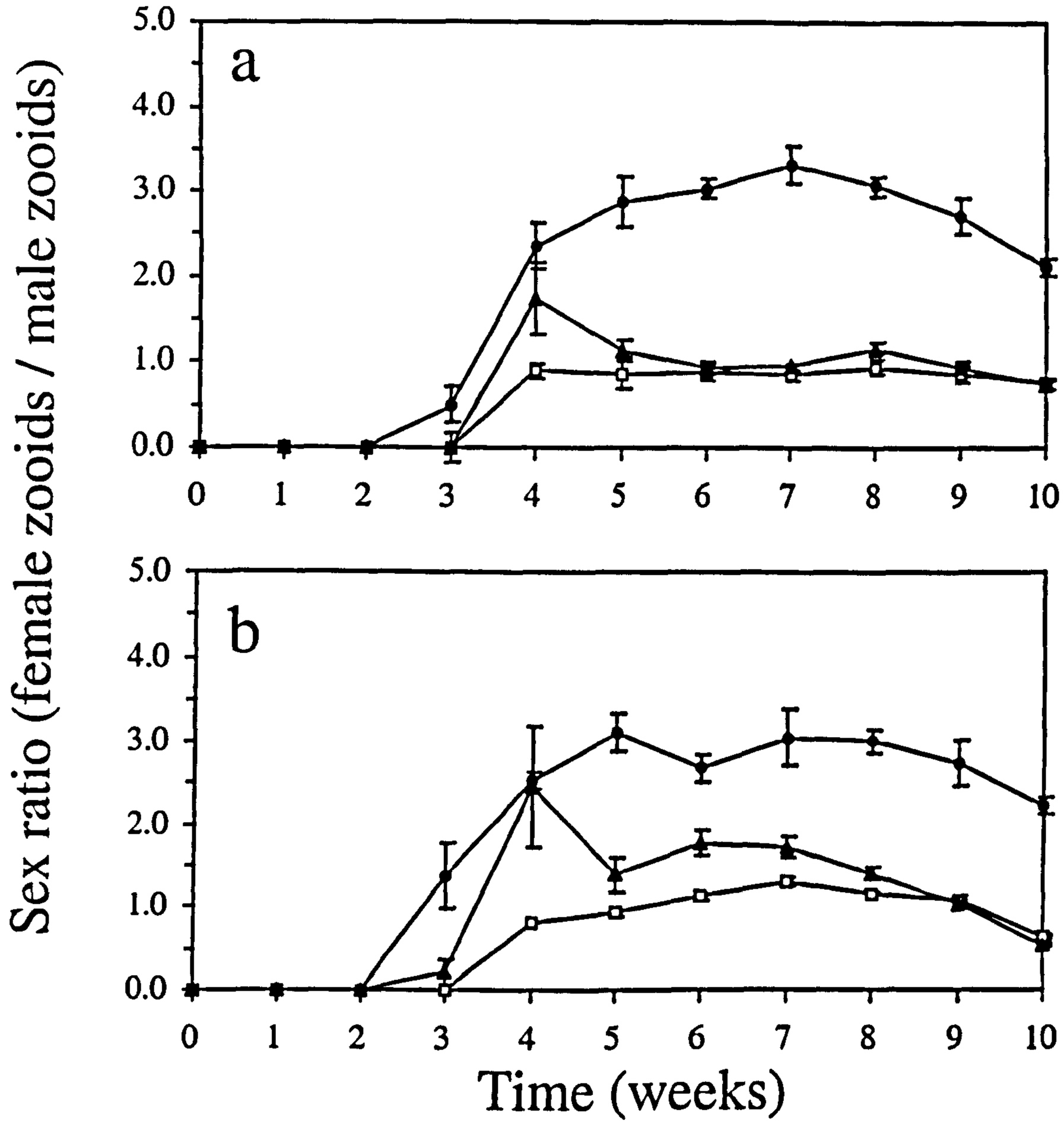


Figure 25: Experiment 5, Sex ratio in colonies of C. hyalina under three different regimes of sperm dosage. Data are mean ± SE. See Figure 21 for the complete legend.

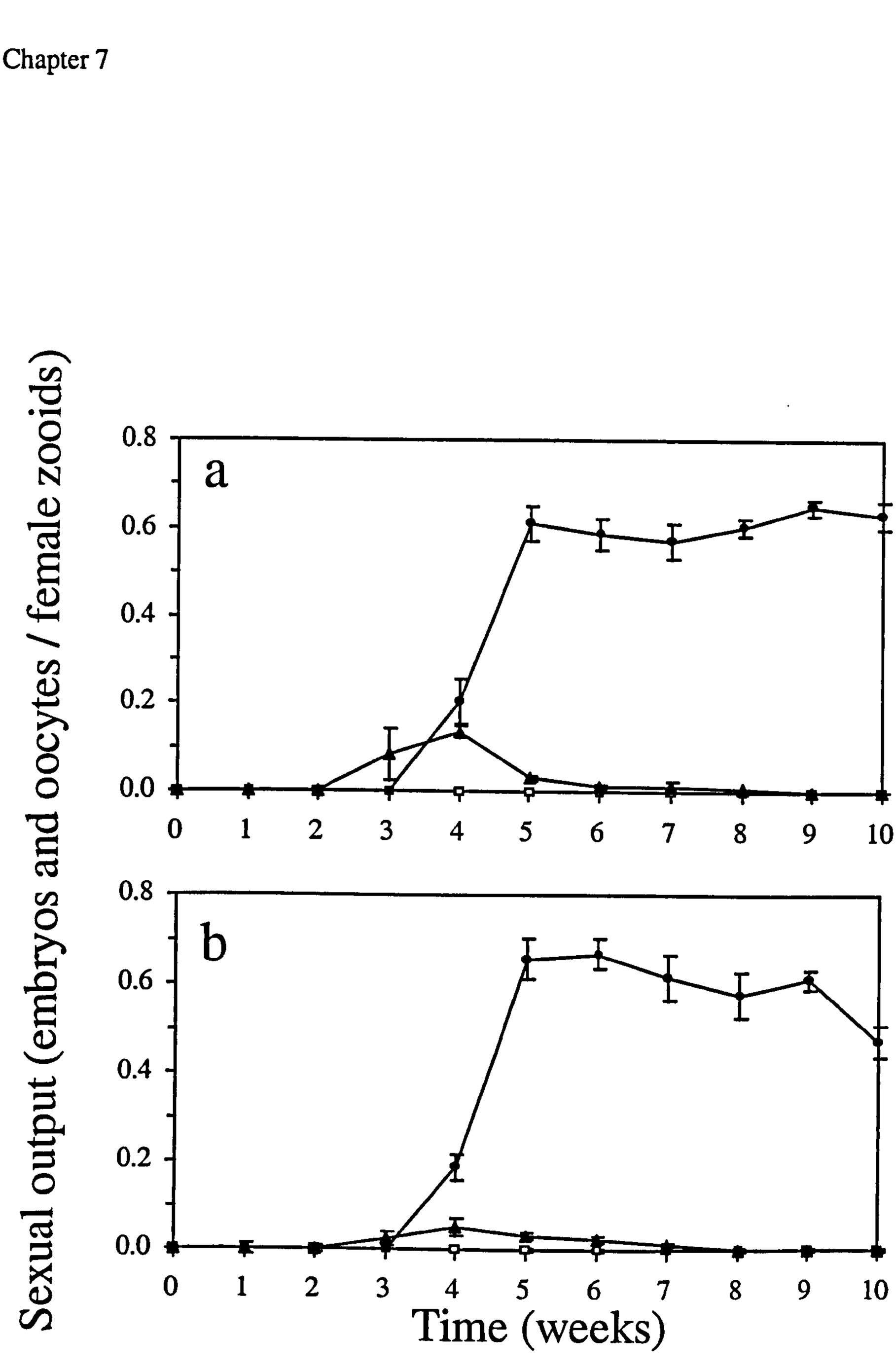


Figure 26: Experiment 5, Sexual output in colonies of C. hyalina under three different regimes of sperm dosage. Data are mean ± SE. See Figure 21 for the complete legend.

Chapter 8:

The consequences of genetic relatedness on inter-colonial fusion.

8.1 Introduction

The phenomenon of fusion between conspecifics (chimerism) is well documented in the literature (see Grosberg, 1998 for a review). Moreover, the underlying allorecognition also is well documented in some marine invertebrates (Bancroft, 1903; Buss, 1982, 1985; Grosberg, 1988). In colonial organisms, allorecognition allows the genet to maintain its identity. However, in certain combinations two conspecific colonies may fuse to form a chimera. Chimerism has been described in a variety of taxa including ascidians (Chadwick-Furman and Weissman, 1995; Rinkevich et al., 1993; Hirose et al., 1994; Bishop and Sommerfeldt, 1999), hydrocorals (Frank and Rinkevich, 1994), hydroids (Shenk and Buss, 1991), corals (Noble and Lee, 1991), sponges (Amano, 1990; Maldonado, 1998) and marine and freshwater bryozoans (Moyano, 1967; Stebbing, 1973; Chaney, 1983; Mukai et al., 1984; Shapiro, 1992, 1996; Craig, 1994; Ishii and Saito, 1995).

Bryozoans are common encrusting organisms in many shallow water marine environments and are found on a wide range of substrata, including rocks, algae and other animals. Aggregations of conspecific bryozoan colonies are found worldwide, commonly occurring on seaweed and on hard substrata. In several species of bryozoans there is a general tendency for genetically related larvae to settle gregariously (Keough, 1984). This result has been associated with colonisation of the substratum by family clusters (Jackson, 1986) and the occurrence of inbreeding (Grosberg, 1987). These settlement patterns agree with predictions based on advantages of minimizing interspecific competition (Stebbings, 1971; Seed and O'Oconnor, 1981; Ryland, 1976), escaping predation (Keough, 1984) and with facilitation of cross fertilisation among simultaneously hermaphroditic colonies (Ryland, 1970; Hayward and Ryland, 1975; Cancino, 1986). However, two important and inevitable consequences of gregariousness linked to philopatry is to increase the relatedness of potential mates (Grosberg and Quinn, 1986) and to increase the chance of contact between adjoining colonies. In marine bryozoans, colonial fusion is possible between parts of the same colony and

between different colonies (autosyndrome and homosyndrome respectively sensu Knight- Jones and Moyse, 1961). Homosyndrome has been observed in Welsh colonies of Celleporella hyalina and Membranipora membranacea (own observations) and has been described in the cheilostome bryozoan Thalamoporella californica (Chaney, 1983). Although homosyndromy is well documented for bryozoans (Moyano, 1967; Stebbing, 1973; Humphries, 1979; Craig, 1994), to date there is little information about colony specificity (allorecognition). Studies in T. californica (Chaney, 1983) had shown that fusion occurs only between zooids of the same colony or between siblings colonies. In this study no fusion was found in non-sibling colonies.

Colonies of the marine bryozoan C. hyalina are epiphytic, commonly occurring on kelps and fucoids (Cancino, 1986). On these substrata, high colony densities occur toward the younger meristematic areas (Stebbing, 1972), the colonies eventually being destroyed by disintegration of the substratum at the end of the fronds (Cancino, 1986). Larvae of C. hyalina are brooded in special chambers for about two-three weeks (DJ. Hughes, see Chapter 3) and then spend between 2 and 24 hours in the water column as planktotrophic larvae (Orellana and Cancino, 1991, Orellana et al., 1996, own observations) before settlement. On the Welsh coast the common substrata for C. hyalina are fronds of brown macroalgae (Cancino, 1986). On these substrata it is possible to find groups of colonies growing close to each other, interactions or contacts between adjoining growing edges being a common feature (Cancino, 1983; own observations). As high numbers of new and adult colonies of C. hyalina are present on their natural substratum, it is possible that this species may be capable of fusion. The objective of the present study was experimentally to evaluate the capacity of colonies of C. hyalina to fuse when they made contact with each other at their growing edges. Fusion was assessed in pairwise combinations of colonies with different genetic relatedness, grown under laboratory conditions. Moreover, observations were made on contiguous colonies growing on their natural substratum, collected in the field.

8.2 Material and Methods

8.2.1 Experimental ramets

Parental clones (A_1, D_1, E_1, J_1, M_1, and Q_1) of C. hyalina were used. Full sib and half sib progeny were experimentally produced by mating those clones. Unrelated clones (N_2 and S_2) were established as above but from larva released from a batch of colonies collected in the same area in October 1996. Artificial propagation was used to produce sufficient copies of the experimental clones (see Appendix 1).

8.2.2 Experimental manipulations and culture conditions

Ramets of each clone were maintained in a 2 l PET with a maximum number of 24 clonemates per bottle (see Appendix 1). Colonies were maintained with a diet of the flagellate chrysophyte Rhinomonas reticulata (formally Rhodomonas baltica, see Novarino 1992). Concentrations of food in the PET bottles was maintained at of about 100 cells·μl^{-1}, recommended by Hunter and Hughes (1983a). In all the procedures, 0.2µm filtered UV treated seawater (hereafter FSW) was used. All the experiments were conducted from December 1998 to July 1999 in a controlled-temperature room at 15-16°C with a 12:12 dark/light photoperiod.

In order to investigate the possible effects of fusion on colony size, in Experiments 2 and 3 additional information was required about colony size and proportion of active zooids per colony. This information was obtained from camera lucida drawings and direct counting of active and recycling zooids in the experimental couples. As a control, ramets of the same clones were maintained in the same PET bottles, but in the absence of contact. This was required in order to avoid possible effects of their contact with the experimental colonies.

8.2.3 Experimental scoring for colonial fusion

To accurately characterize fusion in C. hyalina it was essential to monitor the experimental couples. The pairs of experimental colonies were observed once a week under a stereo microscope (Wild M3). Camera lucida drawings were made of each colony. Using these drawings, additional information about the intercolonial interaction and the angle of contact between the facing zooids in the contact area was addressed. When contact between the growing edges took place, colony borders were examined for the evidence of

morphological fusion. The presence of coalescent zooids (Craig, 1994) was used a conservative criteria for fusion. In the absence of coalescent zooids, different degrees of fusibility were considered under the general category "not fused".

Following intercolonial contact, five types of morphological responses were observed at the growing edges: (a) non-fusion with erect growth, (b) contiguous growth, (c) fusion with coalescent zooids, and (d) non-aggressive overgrowth. In the non-fusion reaction the growing edges of the adjoining colonies stopped the budding of new zooids. In this zone erect growth was characterized by the formation of a ridge between the colonies in contact. This ridge was constituted by basal autozooids or basal male zooids from one colony growing onto a similar zooid in the opposite colony, as if it were a substratum. This response was almost always the first reaction after intercolonial contact and was associated with contact among opposing basal zooids facing one against each other by their distal margins. The overgrowth reaction was confined to small sections of the contact area, where one of the adjoining colonies was in poor condition, dead or recycling autozooids being overgrown by zooids from the healthy colony. As this reaction was rarely found, it was not included as a final experimental score. However, the other three reactions were used to assess the final score of the intercolonial contact at the end of the experimental period. Two final scores were assessed per pair of colonies in contact, each one being derived from observations concentrated on the contact zone, close to the common growing edge. Thus each final score was a combination of the following scores: non-fusion with erect growth (hereafter CB); contiguous growth or colliding colonies (hereafter CC) and fusion with presence of coalescent zooids (hereafter CZ).

8.2.4 Experiment 1: contacts between maternal colonies and their progeny and contacts between daughter colonies

Four ramets of C. hyalina from each of 5 different clones were exposed for two hours to allosperm suspensions from sexually compatible clones. The colonies were then rinsed with FSW and assigned to individual 250 ml glass jars, fed with R. reticulata daily and cleaned every second day. Two conditioned acetate sheets were placed in each container as settlement substrata, one on the bottom and the other against the walls. Contact between adjoining settled colonies and between the maternal colony and daughter colonies were

scored one month after exposure to the allosperm suspensions. Only contacts between pairs of colonies were scored, those among bigger groups were ignored. As self-fertilization is rare in C. hyalina (Hoare et al., 1999; present thesis), all colonies produced by maternal colonies exposed to a single allosperm suspension were considered to be full sib contacts. Daughter colonies produced by maternal colonies exposed to a mixture of allosperm suspensions were considered to be a mixture of full sibs and half sibs.

8.2.5 Experiment 2: isogenic and allogenic contact between small colony fragments

Interaction between adjoining colonies was assessed by attaching two small fragments of acetate, bearing 30-40 basal active basal zooids to a third piece of acetate serving as a common substratum. The colonial fragments were orientated with their growing edges facing each other and initially separated by 500-600 μm (Plate 2 a). Four types of contact were experimentally produced: (a) isocontact or contact between ramets from the same clone; (b) paternal allocontact or contact between paternal and progeny ramets; (c) full sib allocontact; (d) half sib allocontact and (e) unrelated allocontact. The first experimental run used ramets from the clones A_1, E_1 and M_1, sibling ramets of clones produced by mating A_1 with M_1 (clones AM_1 to AM_6) and ramets of sibling clones obtained by mating A_1 with E_1 (clones AE_3, AE_5, AE_8). Ramets of clones N_2 and S_2 were used as unrelated clones. The second run used ramets from clones D_1, J_1 and Q_1, ramets sibling colonies produced by mating Q_1 with D_1 (clones QD_2, QD_4, QD_6) and ramets of sibling clones produced by mating Q_1 with J_1 (clones QJ_6, QJ_9). The same unrelated clones used in the first run were used here. The acetate sheets bearing the experimental pairs of colonies were maintained in plastic histology racks with a maximum number of 12 sheets per rack and two racks per 2 l PET bottle (see Appendix 1). To assess the effect of contact on colony size, the area of smaller colony was divided by the area of the larger one at the end of the experiment, the index therefore having a maximum value of 1.

The colonies and experimental substrata were cleaned twice a week using artist's brushes. All the experimental colonies were fed daily and, with the exception of one week, the seawater was changed every second day. The experimental colonies were maintained in the laboratory for 6 months, during which they were observed at weekly intervals. Early observations involved the entire contact zone, but the final scorings were concentrated on the line of contact and near to the free growing edge. Since two areas of free growing

edges were present in each pair of colliding colonies, two scores were made per pair. As frontal budding hindered observation of basal zooids, contact areas were observed from both their frontal and basal side. Autosyndromy was also studied in this experiment by observing the outcome of contact between zooids within each colony. This was possible because the tendency of any growing colony of C. hyalina to restore circularity through its growing edges coming into contact with each other (Cancino, 1983).

8.2.6 Experiment 3: contact between small colonies with different levels of coancestry

In the present experiment controlled settlement was used to generate contact between full sibs, half sibs, full sibs and half sibs, and between unrelated newly settled colonies. Larvae were obtained by exposing maternal ramets to sexually competent allosperm suspension. Ramets of clones A_1 and Q_1 were used as maternal colonies. In order to assure sufficient numbers of larvae, large receptor colonies between 3-4 cm in diameter were used. Four ramets of A_1 were used, two being exposed to allosperm from M_1 and the others to allosperm from E_1. Similarly, four ramets of Q1 were used, two being exposed to allosperm from J_1 and others to allosperm from D_1. After mating, the recipient colonies were maintained in reproductive isolation until late embryos appeared in the brood chambers. Maternal colonies were maintained in darkness and larval released induced by exposing the colonies to bright light. Larvae were collected using a plastic pipette and transferred to miniature settlement units (<ca. 500 µl), designed to obtain distances of not more that 400 µm between pairs of newly settled larvae. The settlement units were constructed from Eppendorf micro tube lids glued to a transparent plastic tray lids. Small pieces were cut from conditioned acetate sheet and placed in the bottom of each lid and the unit filled with FSW. Larvae released by the first maternal colonies were allowed to settle in the units and their positions mapped. Larvae released by the corresponding second maternal colonies were then allowed to settled on the substrata. Care was taken in order to obtain the second settlement within 4 days of the first. The settlement units were then placed for one week in plastic vessel containing 6 l of FSW. During that time, colonies were fed with R. reticulata and the water maintained under a high aeration regime. The combinations of newly settled colonies generated using this proceeding are summarized in the Table 1.

Once the two required larvae were settled, the piece of acetate was removed from the lids and glued to a second 4.5 x 7.5 cm acetate sheet. A maximum number of 12 acetate sheets

bearing pairs of colonies were placed in plastic histology rack. Glass slides (c.a. 2.4 x 7.5 cm) were used to maintain the acetate sheet in place. Two rack were assigned per 2 l PET bottle as described above, the FSW being changed every second day and R. reticulata supplied daily as food. Once a week, the colonies were cleaned using a soft artist's brush. At the same time the racks and glass slides were replaced for clean ones, and each PET bottle with their corresponding plastic lid, aeration line and air stone cleaned with fresh water. The experimental colonies were maintained in the laboratory for 1 month and the final scoring of each pair of colonies made.

8.2.7 Experiment 4: reproductive stage and fusibility in isocontact

The present experiment was carried out to assess if fusibility between ramets of the same clone is affected by reproductive status of the ramets. Two pairs of small ramets of each of six clones were produced as described in Experiment 2. Six pairs of ramets of each clone were maintained in 2l PET bottles under reproductive isolation. However, the other six couples were maintained in similar bottles but exposed to allosperm suspension. The colonies were maintained under these conditions for 3 months and then fusion was evaluated following the criteria described above.

8.2.8 Experiment 5: fusion and intercolonial integration

Two approaches were used to assess colonial integration between fused colonies. First, a mechanical stimulus was applied to one of the colonies and the response was observed in both colonies. To generate the stimulus an eyebrow hair attached to a piece of plastic supported by a mechanical manipulator was used to carefully disturb protruded lophophores in one of the colonies. An acetate sheet bearing two colonies in contact was placed in a plastic Petri dish with 10 ml of FSW and R. reticulata cells added to the water in order to stimulate lophophore protrusion. Once lophophore activity was observed in both colonies, the hair was carefully brought to the surface of one of the colonies in order to gently touch the protruded lophophores. In preliminary trials, the contact between the hair and the protruded lophophore triggered lophophore retraction. At the same time that protruded lophophores were being stimulated protruded lophophores of the other colony were closely observed. Retraction of protruded lophophores in the undisturbed colony was considered as evidence of intercolonial integration. Three different conditions were studied using five couples in each one; a) 10 couples of fused colonies (presence of coalescent

zooids); b) 10 couples of colliding colonies (colonies in contact but without coalescent zooids) and; c) 10 couples of colonies without physical contact. Using different couples in two experimental conditions: a) couples of fused colonies and b) couples of colliding colonies, a physical stressor was applied to one of the two colonies. Thus, one of the colonies was exposed to desiccation three times for 30 min with 5 minutes of immersion between each desiccation period. Under those conditions C. hyalina normally tends to produce basal males (see Chapter 7). After exposure to stress, the colonies were maintained under standard culture conditions and the presence of basal zooids evaluated after 2 weeks. Presence of basal males only in the stressed colony was interpreted as absence of colonial integration. However, presence of basal males in both colonies was interpreted as colonial integration. Numbers of basal males were counted in 10 1000 x 1000 μ squares (ca. 10-13 basal autozooids) along the ring of new zooids budded beyond the initial growing edge of the colonies after desiccation was applied.

8.2.9 Field observation

Fronds of L. saccharina bearing colonies of C. hyalina were collected in May, June and July 1999 in the Menai Strait at Beaumaris, Anglesey, UK (53°16'S; 4°05'W). Because of frontal budding and opaqueness of the algal substratum, observation of the basal zooids in the contact required the following protocol. Small sections of algal tissue, in total bearing 40 pairs of contagious colonies, were placed in plastic Petri dishes with 5 ml of FSW and maintained in darkness. However, in the June sampling 15 groups of more than 2 colonies were also included. Every day the water was replaced, and R. reticulata cells used as food. Care was taken each day to remove dead and rotten pieces of fronds from the Petri dishes, using a plastic pipette and a soft artist's brush. After 2 weeks the colonies were no longer attached to their substrata and observation of the contact area could be made when the colonies were still alive. As in the previous experiments, scoring was conducted by observing the contact area from the frontal and basal sides of the colonies. Care was taken to avoid damaging the possible intercolonial bonds in the contact area, using a soft artist's brush to manipulate the colonies. Only colonies whose ancestrulae could be recognized were included in the analysis. This precaution was taken in order to avoid including as two or more colonies pieces of just one colony that previously had been fractured and then fused when their zooids made contact.

8.3 Results

8.3.1 General results of colonial interactions

In experiment 2, with small fragments of colonies, growing edges made contact between 4 and 6 weeks after the beginning of the experiments. In Experiment 3, with controlled settlements, contact took place after one or two weeks after settlement. While the colonies continued growing together, the angle of contact between the opposing basal zooids became smaller. Since the angle of contact was small, opposing zooids were now faced by their lateral walls. Once intercolonial contact occurred between opposing basal zooids faced by their lateral walls three different responses were found. In the non-fusion reaction, erect growth was present from the zone of initial intercolonial contact to the end of the common growing edge. This was visible as a long ridge running along the contact area between the two colliding colonies (Plate 3, view c). The ridge was sometimes interrupted by the absence of zooids growing onto zooids of the other colony, leaving free space between both colonies. However, sometimes these spaces were created by the soft brush removing erect zooids during cleaning. In contiguous growth, as the contact angle of the facing zooids became smaller, erect growth was followed by a zone where the growing edge of each colony become unrecognisable (Plate 1, view a). Thereafter, the colliding colonies continued growing as one sheet. Fusion was indicated by production of coalescent zooids, formed by the fusion of buds produced from each colony. The coalescent zooids were basal autozooids or basal males with a "V" shaped skeletal morphology (Plate 1, views b-e). An extension of the zooecium was clearly situated in each adjoining colony and a single shared orifice was present. Thus, in a coalescent autozooid, one digestive tract is seen in the common coelom. However, on a few occasions, two digestive tracts were found (Plate 1, view d). Soon after the appearance of coalescent zooids, the fused colonies continued growing synchronously in the same direction, as one sheet.

8.3.2 Experiment 1: contacts between maternal colonies and their progeny and contacts between daughter colonies

The final scores for this experiment are summarised in Figure 1. In total, 319 intercolonial contacts were scored. They corresponded to contact between the maternal colony and their progeny (30), between full sib colonies (162) and between full and between half sib colonies (127) colonies. In these three categories of contact, more than 75% of responses were those associated with colonial fusion. The score CB, denoting

absence of fusion, was greatest in the contacts between maternal and daughter colonies (22%). Absence of fusion in the contact between full sib colonies and in the contact between full sib and half sib colonies were represented at lower levels (10% and 13% respectively).

8.3.3 Experiment 2: contact between small colony fragments

The growing edges of the experimental colonies made contact between 4 and 6 weeks after the beginning of the experiment. However, presence of coalescent zooids was observed only from the third month, when newly budded basal zooids from the two adjacent colonies began to be aligned by their lateral walls. As in the previous experiments, the same types of morphological responses were observed when the colonies made contact at their growing edges. In the same pair of colonies, it was possible to find two responses, one response being located on one side of the contact zone and other response on the opposite side. In this case, final scores were treated as in the previous experiments and the frequencies of the final score are shown in Figure 2. Indices of colonial size at the end of the experiment are summarized in Table 2.

8.3.3.1 Unrelated contacts

With members of the family AME, 17 pairs of unrelated couples were followed. The most frequent final score was CB-CB (Fig. 2a; 10 couples or 58.8%). Next most frequent final score was CC-CB (Fig. 2a; 6 couples or 35.3%). The final score CC-CC was found only in one colony (Fig. 2a, 5.9 %). Coalescent zooids were never observed in these trials. With members of the family QDJ, 14 pairs of unrelated couples were followed. The most frequent final score was CB-CB (Fig. 2b; 11 couples or 78.6%) and the rest being CC-CB (Fig. 2b; 3 couples or 21.4%). By the end of the experiment both colonies in each pair were similar in size (see Plate 2 c), similar and high indices of colonial size being found (Table 2).

8.3.3.2 Half sib contacts

With members of the family AME, 18 pairs of half sib couples were followed. The frequencies of the final scores are shown in Figure 2. Coalescent zooids were present in the scores CZ-CZ (Fig. 2c; 1 couple or 5.6%) , CZ-CC (Fig. 2c; 3 couples or 16.7%), and CZ-CB (Fig. 2c; 1 couple or 5.6 %). However, colonial bridge or absence of fusion was found

in the scores CC-CB (Fig 2c; 4 couples or 22.2 %) and CB-CB (Fig 2c; 4 couples or 22.2%). Finally, fully coalescent colonies, CC-CC, was found in 5 couples (Fig 2c; 27.8%). In the family QDJ, fully fused colonies or CZ-CZ were found only in one pair of colonies (Fig. 2d; 8.3 %). However, coalescent zooids were present in the scores CZ-CC (Fig. 2d; 2 couples or 16.7%) and CZ-CB (Fig. 2d; 1 couples or 8.3%). Absence of fusion was found in the scores CB-CB (Fig. 2d; 4 couples or 33.3 %) and CC-CB (Fig. 2d; 3 couples or 25%). Contiguous colonies or CC-CC was found in one couple (Fig. 2d; 8.33%). As in contact between unrelated colonies, here similar and high indices of colonial size were found (Table 2).

8.3.3.3 Full sib contacts

From the 15 contacts attempted between full sibs of the family AME, the final score CZ-CZ was found in almost half of the couples (7 couples or 46.7 %, Fig. 2e). Presence of coalescent zooids in the final score was also found in CZ-CC and CZ-CB (6 and 1 colonies or 40% and 6.7 % respectively, Fig. 2e). The score CC-CC was found in only one couple (6.7%, Fig. 2e). The scores CC-CB and CB-CB were not found (Fig. 2e). In the family QGJ, from the 9 contacts staged between full sibs the most represented score (5 couples or 56%, Fig. 2f). The score CZ-CC was also represented (3 couples or 34%, Fig 2f). Only in one couple the score CC-CC was recorded (12%, Fig. 2f). Similarly to results of the previous contacts, contact between full sib fragments of colonies ended up with both colonies of the same size (Table 2).

8.3.3.4 Mother-daughter contacts

From the 18 contacts staged between sibs of the family AME, and ramets fragments of their parental clones, fusion score CZ-CZ was found in 9 couples (50%, Fig. 2g). In addition, the score CZ-CC was also found in 9 couples (38.9%, Fig. 2g). The score CZ-CB, CC-CB, and CB-CB were not recorded (Fig. 2g). Finally, the score CC-CC was found in 2 couples (11.1%, Fig. 2g). In the 19 contacts staged between sibs of the family QDJ and their paternal clones, the score CZ-CZ was also the most represented score (couples or 73.7%, Fig. 2h). All the other experimental couples ended up with the CZ-CC score (4 couples or 26.7%, Fig. 2h). Differing from all the previous colonial contact, at the end of the experiment indices of colonial size were lower, one colony being bigger than the other one (Table 2). In all the cases, the colonies generated from

the parental fragments were bigger than the colonies generated from the daughter ones (see Plate 2, view e).

8.3.3.5 Self contacts

From the 13 contacts staged between self-fragments involving members of the family AME, the fusion scores CZ-CZ and CA-CC were the only recorded scores 9 (61% and 39% respectively, Fig. 2i). Similarly, these scores were the most common when self-contact were conducted between fragments of the same genotype. However, the score CC-CC was recorded in one couple (9% Fig. 2j).

8.3.4 Experiment 3: contact between small colonies from manipulated settlements

The initial space in between paired colonies was smaller (Plate 3) than in the previous experiment with vegetatively propagated colonies. Consequently, the growing edges of most of the colonies made contact by the end of the second week. However, in a few cases, as the growing edges were facing different directions, contact did not occur until after the end of the fifth week. Usually, however, the required time to obtain zooids of both colonies in contact, with their lateral walls facing in an appropriated angle for fusion, was shorter than in the previous experiment. The final scores were treated as in the previous experiments and they are summarized in Figure 3.

8.3.4.1 Contact between newly settled full sib colonies

For the 12 full sib couples of newly settled colonies, the final contact scorings are summarized in Figure 3a. The most frequent scores were those associated with colonial fusion. The final score CZ-CZ, denoting absence of colonial fusion was not recorded in contact between full sib colonies (Fig. 3a).

8.3.4.2 Contact between newly settled half sib colonies

From the 12 couples of newly settled half sib colonies, the 6% of the contacts ended up with a final scoring denoting absence of colonial fusion (CB-CB, Fig. 3b). The scores CZ-CZ, CZ-CC and CZ-CB related with colonial fusion were recorded in 46, 15 and 17% of the contact respectively (Fig. 3b).

8.3.4.3 Contact between newly settled unrelated colonies

For the 12 couples of unrelated contact between settled colonies, the scores CZ-CZ and CZ-CC denoting colonial fusion were not recorded (Fig. 3c). However, the score CB-CB denoting absence of colonial fusion was recorded in 17 colonies (36%, Fig. 3c).

8.3.5 Experiment 4: reproductive stage and fusibility in isocontact

The final results of the present experiment are summarized in Table 3. Fusion was observed in colonies maintained in reproductive isolation as in those exposed daily to allosperm suspensions.

8.3.6 Experiment 5: fusion and integration

8.3.6.1 Mechanical stimulation

Once the physical stimulus was applied, retraction of protruded lophophores in both colonies was the most common response when the couples were fused or colliding (Table 4). Retraction of the lophophores in only the stressed colony was recorded in low percentages (Table 4). However, when the stimulus was applied in one of the two colonies growing close but not in contact, retraction of protruded lophophores was recorded in similar percentage in the stressed and unstressed colonies (Table 4).

8.3.6.2 Basal male production

Proportion of basal males to basal autozooids in the sampling areas of the stressed and control areas are summarized in Table 5. When the colonies were not fused and in the absence of contact, production of basal males was significantly higher in the experimental colonies exposed to desiccation. However, when the colonies were fused basal males were produced in both colonies, forming a ring close to the growing edge of the colonies, and corresponding to the previous growing edge at the moment that the stress was applied (Plate 3, views e and f). Basal males were recorded in all the stressed colonies and in all the control colonies from the fused treatment (Treatment 1). However, in colliding colonies (Treatment 2), basal males were recorded in only 3 of the control colonies. Finally, in colonies without contact (Treatment 3), basal males were recorded in only 2 control colonies. No significant differences in the proportion of basal males to basal autozooids between stressed colonies and the contiguous control colonies were found in

Treatment 1 (Table 5). However, significant differences were found in Treatment 2 and Treatment 3 (Table 5), with.11 and 45 times more basal males to basal autozooids recorded in control colonies from Treatment 1 than in control colonies from Treatment 2 and Treatment 3 respectively (Table 5).

8.3.7 Observation on wild material

All the pairs of contiguous colonies analyzed remained attached, one to the other, once their natural substratum was removed. Direct observations in the contact area did not reveal any presence of coalescent zooids. When contiguous growth was found, this was fragile and confined to sections of a dominant erect growth area. Therefore, the common morphology in the contact zone in wild couples was erect growth, and small sections of partial overgrowth. Both responses held colonies together even after removal of the substratum. The same findings were observed once the common substratum was removed from groups of 3 or 4 contiguous colonies. No coalescent zooids were found in the contact area between such colonies. However, coalescent zooids were found within each individual colony. These zooids occurred where edges of such colonies had grown into contact. As in the laboratory-reared colonies, the majority of such coalescent zooids were found in the distal part near the ancestrulae, where the growing edges of the colonies first made contact. A morphology similar to the erect growth between two colonies was found within damaged colonies. This corresponded to expansions of the damaged frontal zooidal wall along the plane of fracture, producing calcified elevations similar to those recorded in the erect growth (Plate 4).

8.4 Discussion

8.4.1 Coalescent zooids and angle of contact

The results of the present study show that when two colonies of C. hyalina made contact with each other at their natural growing edge, fusion could take place. Coalescent zooids were strongly associated with intercolonial fusion (i.e. manipulated allocontact and isocontact) and fusion between growing edges within colonies (i.e. natural isocontact). Production of coalescent zooids between fused colonies is in agreement with records for Fenestrulina sp. (Craig, 1994). The presence of coalescent zooids therefore seems to be an appropriate method for assessing fusion in bryozoans of similar morphology to Celleporella and Fenestrulina. Moreover, production of coalescent zooids depends on an appropriate angle between the opposing basal zooids (i.e. autozooids and males) at the contact area. This angle (< 80°) was obtained at different times after the colonies made contact, when newly budded opposing zooids were aligned with their lateral walls in parallel. Angle of contact has been reported to influence the outcome of interspecific interactions (Jackson, 1979; Karande and Swami, 1988; Turner and Todd, 1994).

Evidently, therefore, fusion in C. hyalina it is not an instantaneous phenomenon. This suggests that any future attempt to assess intercolonial fusion in encrusting bryozoans should include time of contact as an experimental variable. In the present study the effect of the angle of contact was accommodated by duration of the experiments. In Experiment 2, the couples were maintained together on the same substratum for 6 mo. As the colonies in this first experiment made contact after about 1-2 mo, they remained in physical contact for about 4-5 mo. In Experiment 3 with controlled settlement, the distance between the newly settled colonies was reduced. Similarly, in Experiment 4 concerning the effect of reproductive status on fusion, the problem was tackled by reducing the initial gap leave between the growing edges of the colonies. Therefore, by the end of all the experiments when the final scoring took place, the appropriate angle between the opposing zooids for production of coalescent zooids was achieved.

Absence of fusion between maternal colonies and their progeny in Experiment 1 can be explained by the unsuitable angle between the basal zooids at the contact line. Contact between big maternal colonies and their offspring would need more time to obtain appropriate curvature for the required angle. As the progeny in this experiment were

needed for genetic fingerprinting, it was not possible to test this hypothesis. On the other hand, the highest values of fusion occurred between daughter colonies. This could be explained by the small size of these colonies. Small colonies need only a few days to obtain the required angle between the opposing zooids along the contact line (own observations). Absence of fusion in some of the experimental couples could be explained by either inappropriate angle or lack of histocompatibility between these colonies.

8.4.2 Colony size

In the present study, the initial sizes of colonial fragments and the newly settled colonies were not different at the beginning of the experiments. This precaution was taken because absolute and relative size has been reported to influence the outcome of interspecific interaction (Buss, 1980, 1982; Winston and Jackson, 1984; Harvell and Padilla, 1990; Stocker, 1991; Nadakumar and Tanaka, 1997). Thus the outcome of the intercolonial contact (i.e. fusibility) in the present study cannot be attributed to differences in colonial size. Similar colony sizes at the end of the experiment in fused colonies (Experiment 2) suggest a lack of deleterious effects of the interaction. However, the size asymmetry denoted by the lowest indices found in contact between fragment of the parental colony and fragments of their progeny suggests deleterious effects.

8.4.3 Colonial relatedness

Fusibility was shown be dependent upon colonial relatedness. Presence of coalescent zooids were found in the zone of contact between maternal colonies and their progeny, as well as between full sib and half sib colonies, and when two growing edges of the same colony made contact with each other. However, coalescent zooids were not found in the contact between unrelated colonies. Fusion between related colonies are in agreement with results for T. californica (Chaney, 1983), where fusion occurred only between zooids of the same colony or between sibling colonies.

The results of Experiment 2 are in agreement with the results of the Experiment 1. As fusion was found only in intercolonial contacts between related colonies and was absent in unrelated contacts, these results can be adequately explained as the consequence of histocompatibility between colonies. The same argument can be used in the experiment

with manipulated settlements (Experiment 3), where fusion again was found in contacts between related but not between unrelated colonies.

Low indices of colonial size, when contacts were originated between the parental and daughter fragments strongly suggests a deleterious effect of the interaction. Since in C. hyalina colony size is positively related with reproductive output these results clearly show negative effect of fusion on fitness. The possibility of inter-individual germ cell parasitism has been proposed in fused chimeras (Buss, 1982). Moreover, experimental evidence suggests that germ cell and somatic parasitism may be possible in chimeric individual (Rinkevick et al., 1993; Saito, et al., 1994; Rinkevich, 1995; Chadwick-Furman and Weissman, 1995; Pancer, et al., 1995; Stoner and Weissman, 1996). In colonial ascidians, germ-somatic parasistims is manifested by colony resorption or allogeneic resorption of one partner of a chimera, which is controlled by a fusibility locus (Saito et al., 1994). The results of the present experiment are in agreement with this finding, suggesting an important role of kin recognition by larvae at the moment of settlement. Because of the deleterious effects of the fusion on the daughter colonies, and the absence of fertilization between maternal and their siblings (see Chapter 7), larvae should avoid settlement close to the parental colonies. These conditions could be important for the evolution of mechanisms of allorecognition, allowing organisms to maintain their genetic identity. These mechanism should play an important role if contact between closely related colonies, which is promoted by gregarious settlement, takes place. However, kin-larvae of C. hyalina tend to have a similar settlement pattern in the presence of a parental colony, a sib colony or clear substratum (personal unpublished data). In these experiments physical elements (i.e. light and water regime in the experimental arena) were more relevant in the spatial determination of settlement.

Since a chimeric organism may express greater physiological capacity than either a single colony component, chimerism among related colonies in the absence of parasitism could be ecologically advantageous. Despite the fact that, in the present study, total resorption of one partner by the other one was not found, prevention of colonial growth after fusion clearly affects the fitness of the depressed colony. This is because in bryozoans colony size is directly proportional to the reproductive output (Jackson and Wertheimer, 1985) Finally, fusion among fragments of the same colony may play an important role allowing the

recovery of integrity after colonial fragmentation, an event common in colonies growing on their natural substratum (personal observation).

8.4.4 Reproductive stage and absence of fusion between wild colonies

The results of Experiment 4 show that fusibility it is not related to the reproductive status of the colonies on contact. Although in the field, colonies of C. hyalina are exposed to allosperm, the results of this experiment suggest that no compromise exists between reproduction and fusibility. As this experiment concerned only isocontact, these results cannot be extrapolated to situations of allocontact. However, this finding suggests that under natural conditions fusion in isocontact could play an important role in maintaining the integrity of fractured colonies. On their natural substratum, fractures in colonies of C. hyalina are a common phenomenon. Moreover, coalescent zooids have been observed within wild colonies of C. hyalina, where they were associated with fracture scars (own observations).

Finally, the absence of coalescent zooids along the contact zone in all the wild groups of colonies analysed suggests that intercolonial fusion it is not frequent in the field. The fact that such colonies remained attached to each other after the algal substratum was removed can be explained by the mechanical bonds formed by erect growth and growth of one colony over the other. As larvae of C. hyalina are photo-positive just after release (Ryland, 1960) and they expend between 2 to 24 h in the water before metamorphosis (Orellana and Cancino, 1991; Orellana et al., 1994, own unpublished results) it is highly likely that they settle in a different place from their maternal colony. Absence of preferential settlement close to a related adults (i.e. maternal colony) or to newly full sib and half sib settled colonies has been demonstrated in manipulated settlement experiment in C. hyalina (own unpublished results). Future studies are required to assess genetic relatedness between colliding colonies in their natural substratum in the field and the range of larval dispersion. To date the only work with wild colonies of the Welsh C. hyalina on their natural substratum suggest that genetic differences are found at small (ca.<10 km) scales of distance (Goldson, 1998).

8.4.5 Physiological integration

The absence of differences between fused, not fused, and non-touching colonies were caused by vibration produced when the manipulator was triggered. This was enhanced by the high sensitivity of protruded lophophores to external perturbations such light and vibrations (own observations). Vibrations propagated through the water were responsible for lophophore retraction in the colony directly exposed to the physical stimuli, as in the other colony. However, in the experiment with desiccation stress production of basal males in the non-stressed colony of the fused pairs strongly suggests that such colonies are physiologically integrated.

The above results suggest that fusion in C. hyalina is strongly dependent on genetic relatedness. Moreover, fused colonies are physiologically integrated. These results are in agreement with neural integration studies on bryozoans (Shapiro, 1996). These findings of physiological integration between colonies, seem to be more robust that previous examples of fusion in bryozoans where the presence of pore plates between colonies has been used as evidence for colonial integration (Moyano, 1967; Stebbing, 1973; Humphries, 1979; Chaney, 1938; Shapiro, 1992; Craig, 1994).

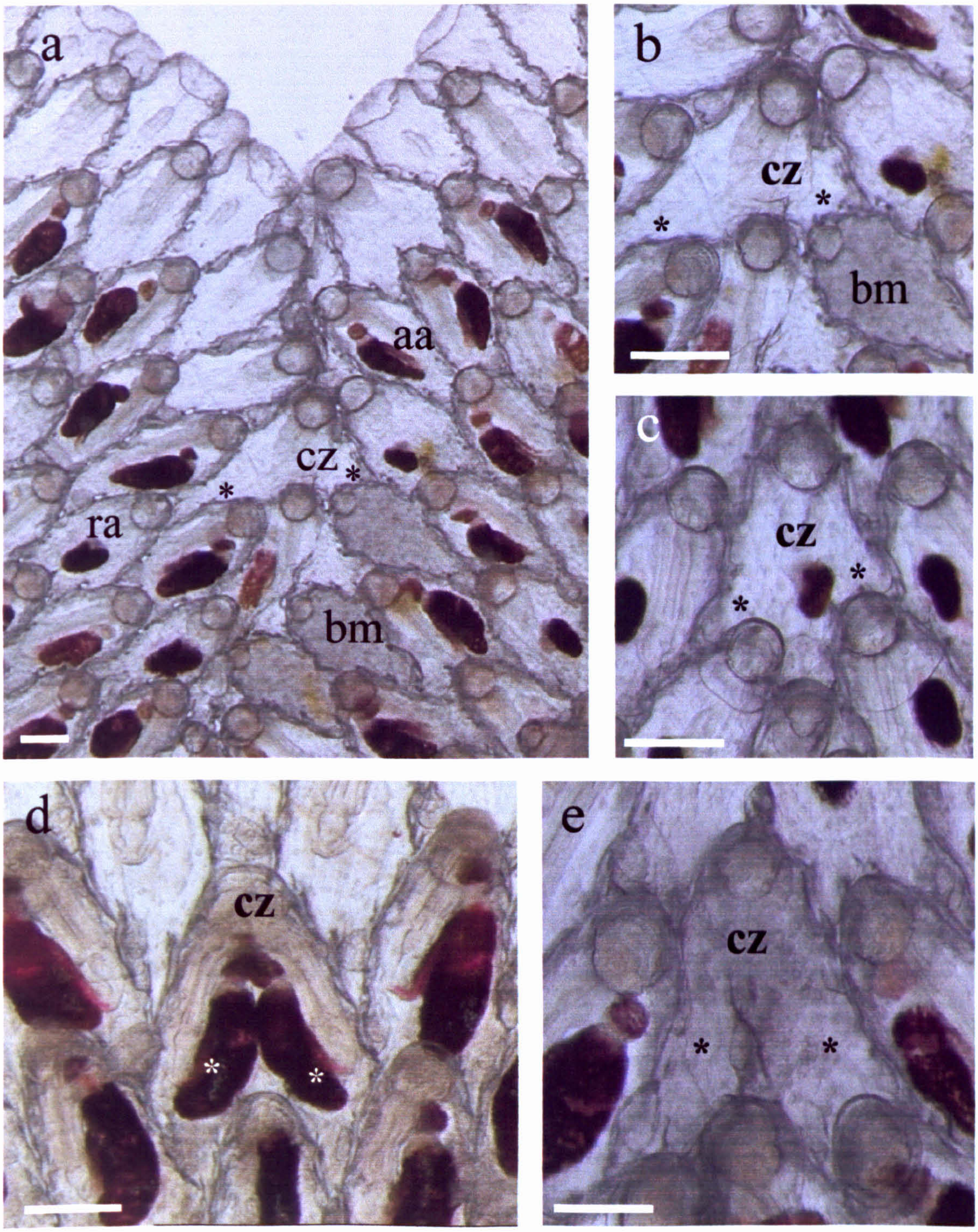


Plate 1: Intercolonial contact in Celleporella hyalina under laboratory manipulated conditions. (a) general view of the contact area between two growing edges of the same genotype; (b), (c), (d) and (e) showing zooids in this area. In (a) basal male zooids (bm), active autozooids (aa), (recycling autozooids (ra) and coalescent zooids (cz) are shown. In (b) the coalescent autozooid is in process of formation; in (c) the coalescent autozooid is recycling; in (d) the coalescent autozooid bearing two complete polypides; and in (e) the coalescent zooid is a basal male. The projections of zooecium from the coalescent zooids in each colony are indicated by (*). In all views scale bars are 100 μm.

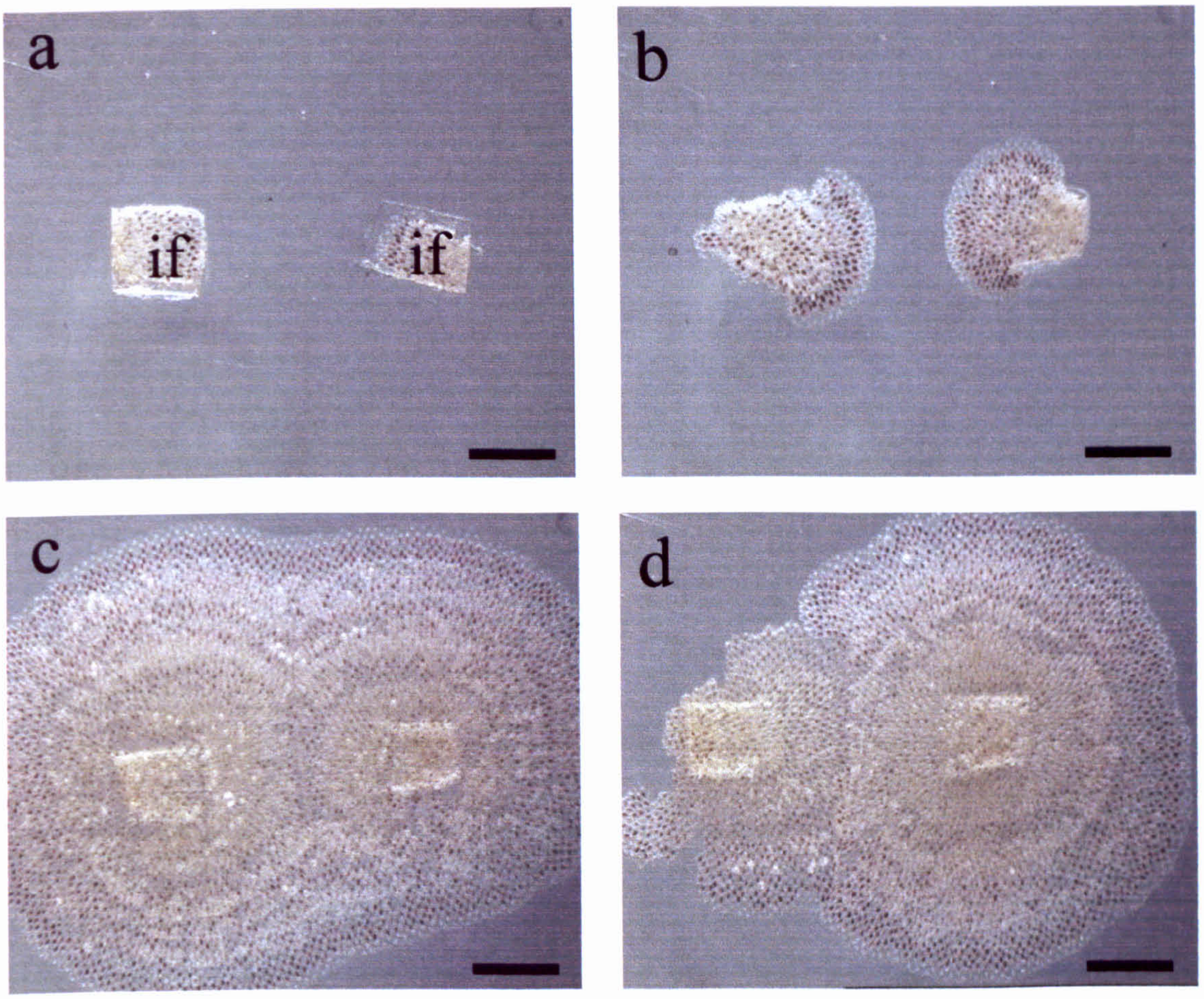


Plate 2: Different stages on the Experiment 2, contact between small colony fragments. In (a) the initial colonial fragments (if); in (b) colonies growing in the common substratum; in (c) and (d) colonies in contact at the end of the experiment. In (c) the contact took place between fragments of the same genotype and in (d) between a maternal and their daughter. Scale bars are 200µm.

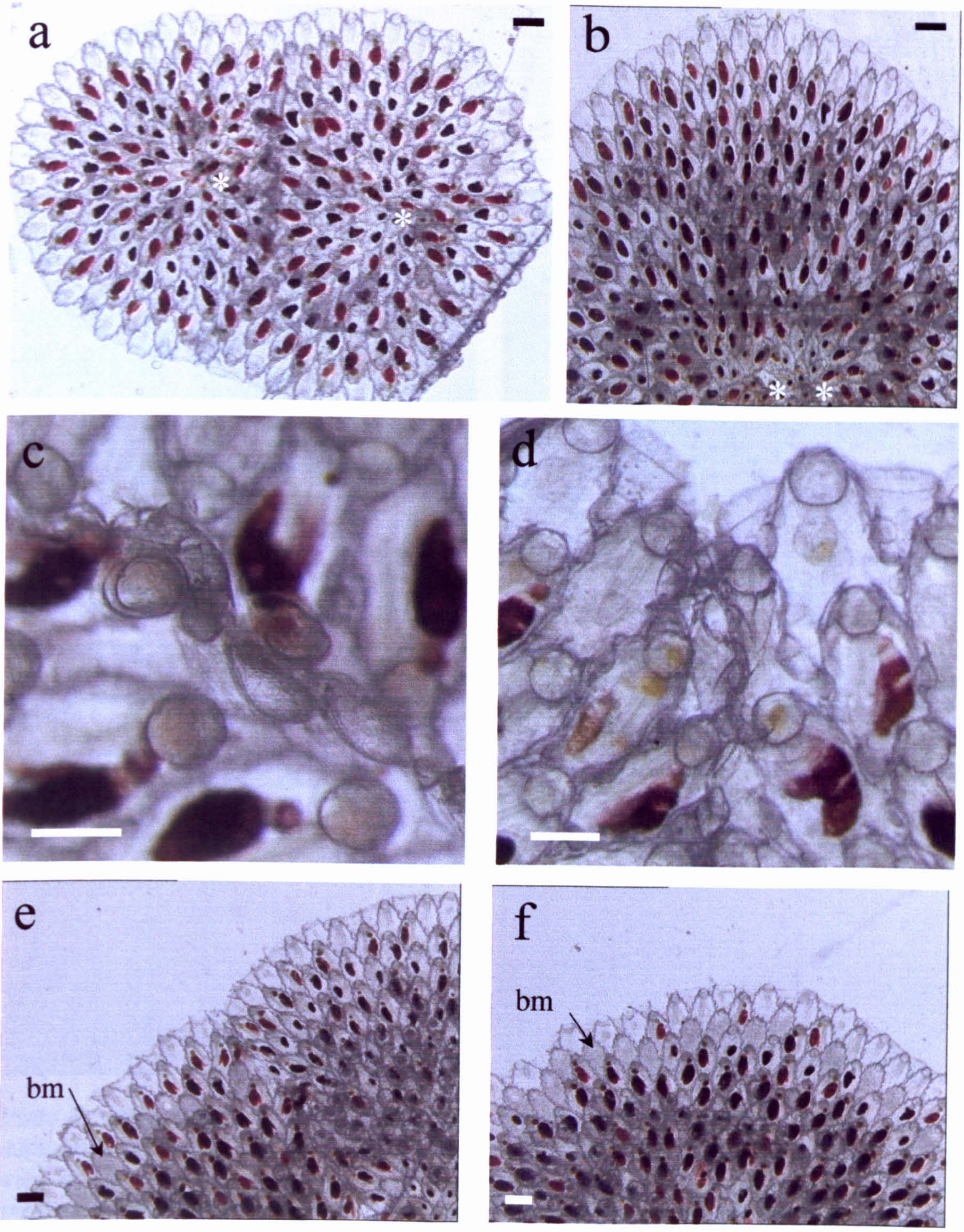


Plate 3: Intercolonial contact in Experiment 3, contact between small colonies from manipulated settlements. General views of the two sexually immature colonies 6 wk (a) and 8 wk (b) after settlement. In (c) the erect growth found in the contact area when opposing basal zooids facing one against each other by their distal margins. In (d) the erect growth disappears giving place to a more smooth contact area before the appearance of the coalescent zooids. In (e) basal males (bm) are only presents in the stressed colony of unfused colonies and in (f) presence of a ring of basal males in both fused colonies. In all the views the scale bars are 200 μm and (*) represents the ancestrulae.

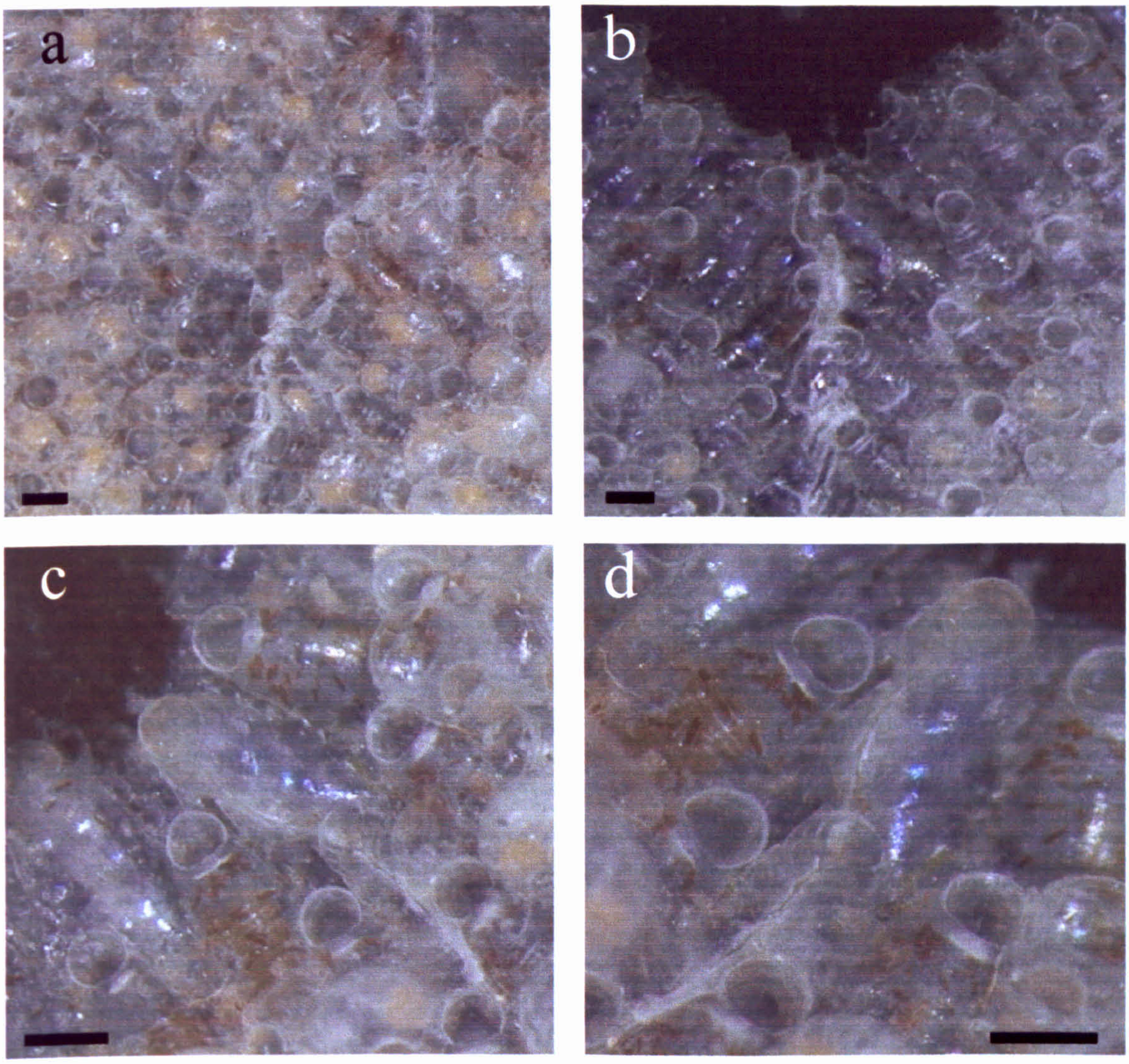


Plate 4: Intercolonial contact in Celleporella hyalina colonies growing on their natural substratum.(a) general view of the contact area originated by three colliding colonies. (b) a view of the contact area close to the growing edge of two colliding colonies showing the presence of the characteristic erect growth and absence of coalescent zooids of non-fused colonies. (c) and (d) erect growth resembling formation produced within a single colony as consquence of calcification along the fractured skeletal walls. Scale bars are 100 μm.

Table 1: Experiments1, Pairs of newly settled colonies of C. hyalina with different levels of coancestry. Full sibs ancestrulae were generated by exposing ramets from each of the maternal clones to allosperm suspensions from the first parental clone. In the mother half sib contact, one ancestrulae was generated by exposing ramets from each of the maternal clones to allosperm suspensions from the first parental clones and the other generated by exposing ramets from each of the same maternal clones to allosperm suspensions from the second parental clone. In the unrelated contact one ancestrulae was generated by exposing ramets from each of the maternal clones to allosperm suspensions from the first allosperm parental clone and the second ancestrulae was generated by wild parental colonies collected on May of 1999 from Menai Bridge.

Relatedness	First parental clone	Second parental clone	Maternal clone	N° of pairs
Mother full sib:	M_1	-	A_1	12
	E_1	-	A_1	12
	Q_1	-	D_1	12
	Q_1	-	J_1	12
Mother half sib:	M_1	E_1	A_1	12
	E_1	M_1	A_1	12
	Q_1	D_1	J_1	12
	D_1	Q_1	J_1	12
Unrelated:	M_1		A_1	12
	E_1		A_1	12
	Q_1		D_1	12
	Q_1		J_1	12

Table 2: Experiment 2, Effect of inter colonial contact on final colony size. The following indices were obtained dividing the area of smaller colony by the area of the larger one at the end of the experiment (i.e. 6 mo). The index therefore having a maximum value of 1. Five treatments were studied: contact between fullsib colonies (Treatment 1); contact between half sib colonies (Treatment 2); contact between paternal and daughter colonies (Treatment 3); contact between fragment of the same genotype (Treatment 4); and contact between unrelated colonies (Treatment 5).

	Family AME	Family QDJ
Treatment 1:	0.95 ± 0.03 (n=12)	0.93 ± 0.04 (n=9)
Treatment 2:	0.99 ± 0.06 (n=18)	0.93 ± 0.06 (n=11)
Treatment 3:	0.54 ± 0.28 (n=18)	0.66 ± 0.33 (n=14)
Treatment 4:	0.92 ± 0.03 (n=18)	0.93 ± 0.06 (n=11)
Treatment 5:	0.91 ± 0.05 (n=18)	0.88 ± 0.08 (n=14)

Table 3: Experiment 4, Percentage of fused and non fused colonies of C. hyalina for colonies maintained under two reproductive regimes. In Treatment 1, 6 couples in isocontact were exposed daily to allosperm. In the Treatment, 6 couples in isocontact were maintained under reproductive isolation. The final scoring were assessed two mo after the begining of the experiment and fused colonies were considered those where presence of coalescent zooids were recorded.

	Fused colonies	Unfused colonies
Treatment 1: (n= 6 couples)	100 %	0%
Treatment 2: (n= 6 couples)	100 %	0%

Table 4: Experiment 5, Location of lophophore retraction as response to a mechanical stimuli. Three experimental treatments were studied: fused colonies (Treatment 1), colliding colonies (Treatment 2) and colonies growing close into the same substratum but without physical contact between them (Treatment 3). The stimulus was applied in one of the colonies and lophophore retraction recorded in both colonies. In all the treatments both colonies in each couple were originally fragments of the same genotype, then potentially able to fuse.

	Location of the lophophore retraction		
	only in the stressed colony	in both colonies	In the unstressed colony
Treatment 1:	13.33%	86.67%	0%
Treatment 2:	6.67%	93.33%	0%
Treatment 3:	40.00%	60.00%	0%

Table 5: Experiment 5, Proportion of basal males to basal autozooids in colonies of C. hyalina after a desiccation stress was applied. In Treatment 1, the colonies were fused (i.e. presence of coalescent zooids), in the Treatment 2 the colonies were colliding (i.e. growing edges in contact but without presence of coalescente zooids), and in Treatment 3 the colonies were into the same substratum but without contact along the growing edges. Desiccation was applied only in one of the two colonies and the proportion of basal males to autozooids assesses in the stressed and the unstressed or control colony. In all the treatments colonies were originally fragments of the same genotype. Data are mean ± SD, and were compared using a Mann-Whitney test data.

	stressed colony	Control colony	P
Treatment 1: (n= 10 couples)	0.102 ± 0.092	0.089 ± 0.092	>0.05
Treatment 2: (n= 10 couples)	0.113 ± 0.092	0.008 ± 0.027	<0.0001
Treatment 3: (n= 10 couples)	0.102 ± 0.078	0.002 ± 0.011	<0.0001

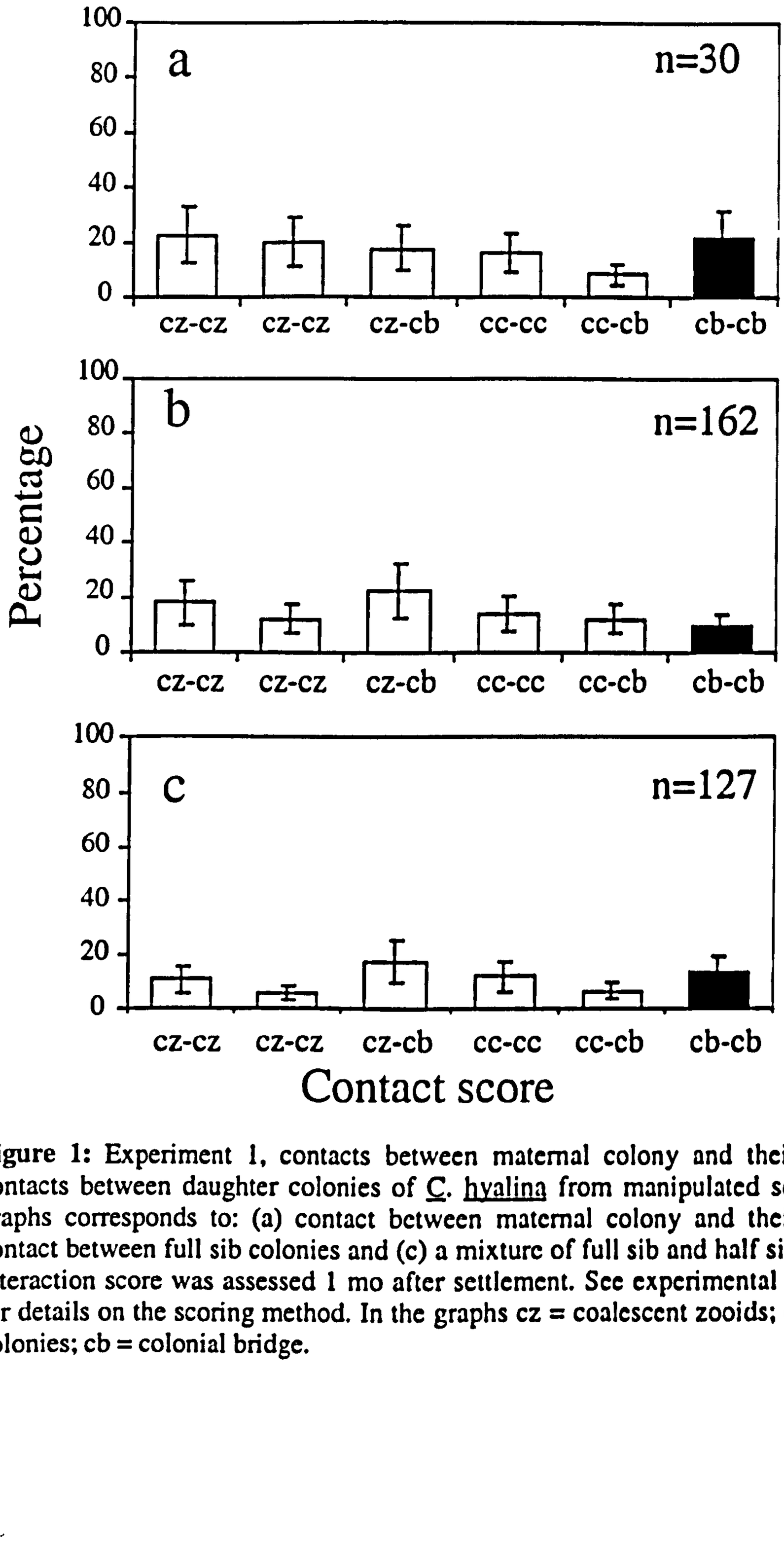


Figure 1: Experiment 1, contacts between maternal colony and their progeny and contacts between daughter colonies of C. hyalina from manipulated settlements. The graphs corresponds to: (a) contact between maternal colony and their progeny; (b) contact between full sib colonies and (c) a mixture of full sib and half sib colonies. The interaction score was assessed 1 mo after settlement. See experimental scoring section for details on the scoring method. In the graphs cz = coalescent zooids; cc = coalescent colonies; cb = colonial bridge.

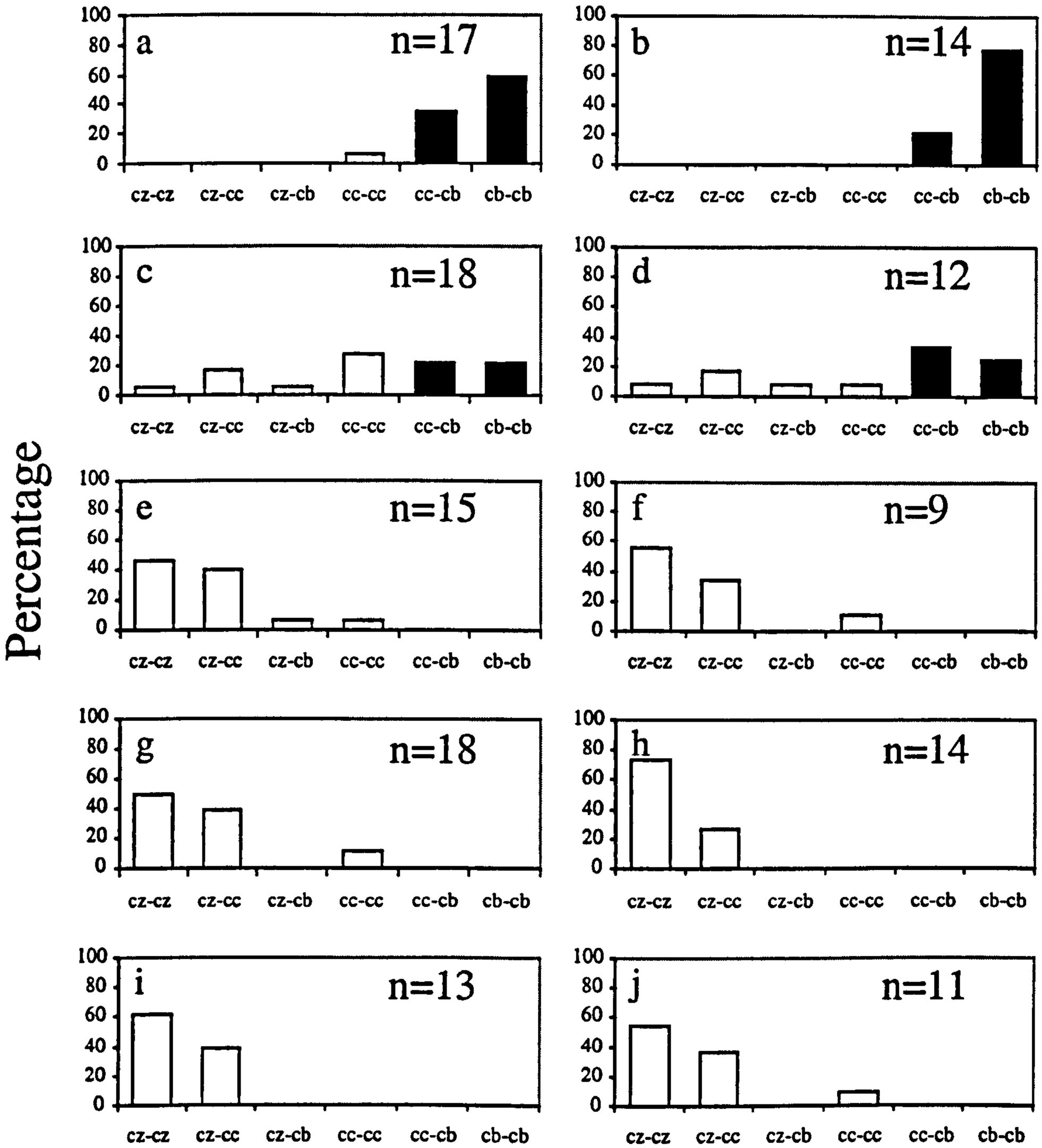


Figure 2: Experiment 2, contact between small colony fragments of C. hyalina. The graphs corresponds to: (a) and (b) contact among unrelated colonies; (c) and (d) contact among half sib colonies; (e) and (f) among full sib colonies; (g) and (h) among the experimental clones the corresponding parental clones; and (i) and (j) self contact. The interaction score were assessed at the end of the experimental period. See Figure 1 for a complete explanation of the contact scoring.

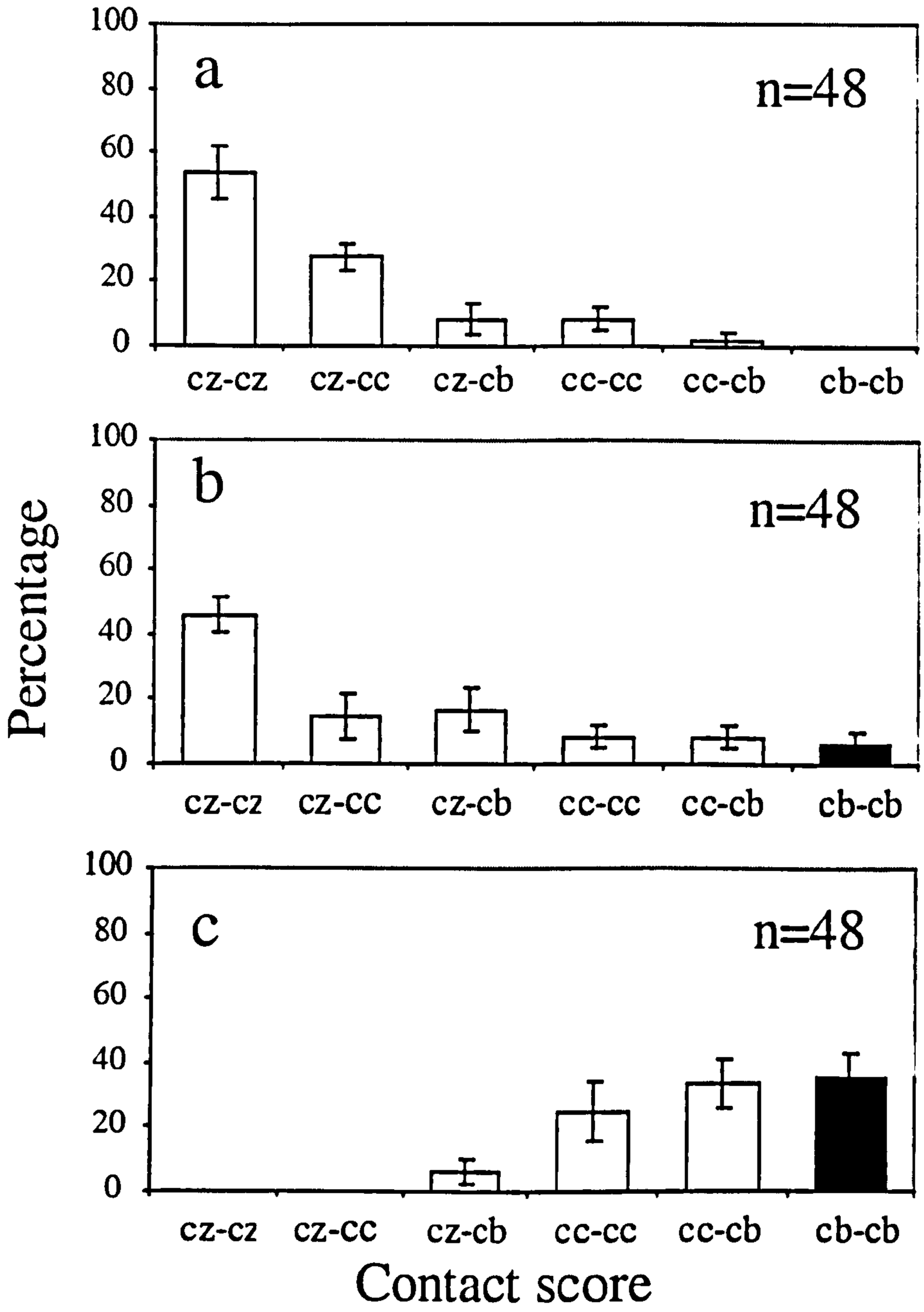


Figure 3: Experiment 3, contact between small colonies of C. hyalina from manipulated settlements. The graphs correspond to: (a) full sib colonies; (b) half sib and (c) unrelated colonies. The interaction score was assessed 2 mo after settlement. See Figure 1 for a complete explanation of the contact scoring.

Chapter 9
General discussion

9.1 Sperm release and sperm longevity

In the present study, sperm release was found to be triggered by a dark-light reaction (see Appendix 2). This could be a mechanism evolved to synchronize pulsed sperm release, enhancing the chances of reaching a receptor colony at higher concentration. A positive relationship between sperm concentration and fertilization success has been demonstrated in the present study. A similar relationship has been found in the compound ascidian Diplosoma listerianum (Pemberton, personal comunication). Moreover, half-life of about 1-2 h (Chapter 2) suggests the existence of a short temporal window of sperm dispersall for C. hyalina. This, together with restricted larval dispersal, should enhance the exploitation of localized areas by genetically related colonies due to low levels of gene flow. This is in agreement with evidence obtained using RAPDS analysis, where highly structured population genetics characterized relatively small spatial scales (Goldson 1998).

9.2 Fertilization success

Examination of results from different experiments in this study strongly suggests that fertilization success can be affected by the capacity of receptor colonies to protrude their lophophores (see Chapter 4). These results strongly suggest that feeding activity is associated with sperm entrance. The present study suggests that the feeding activity of the zooids (i.e. active zooids or recycling zooids) situated beneath the brood chamber influences the location of the growing oocytes, as these are always concentrated in areas within the colonies bearing active zooids. Moreover, abortions were associated with areas in the colonies where, under deleterious conditions, massive zooidal degeneration took place. This suggests that the extra-embryonic nutrition in the ovicells depend on energy allocated from neighboring autozooids to the female zooids.

Results from all the experiments described in the present study strongly suggest the prevalence of outcrossing in C. hyalina. These results are in agreement with results of studies on the Chilean C. hyalina (Cancino et al., 1991). The few circumstances of self-fertilization are in discrepancy with previous studies of Welsh C. hyalina (Hunter and Hughes, 1993c) where self-fertilization was recorded and associated with abortion. Since

in the present study growing oocytes were not recorded in colonies maintained under reproductive isolation, abortion could not be recorded. Self-fertilization has been also recorded in North American C. hyalina (Yund and McCartney, 1994). Since in Hunter and Hughes study (1993c) genetic validation of self-fertilization was not made, presence of progeny in these colonies can be explained by unwanted sperm contamination. Moreover, abortion can be linked to degeneration of the autozooids beneath the brooding chamber bearing the progeny. However, discrepancies with the Yund and McCartney study (1994) could be consequences of comparisons between separates species. Prevalence of outcrossing in the Welsh C. hyalina is in agreement with studies of Hoare (Hoare et al., 1999). Evidence obtained using matings of receptor colonies with sperm of different genetic relatedness (see Chapter 7) indicates a strong genetic component in mating success which favour mating among unrelated and half sib colonies. This finding may be attributed to avoidance of deleterious effects associated with inbreeding (Grosberg, 1987; Hunter and Hughes, 1993c; Cohen, 1996).

9.3 Sperm storage and intracolonial movement

Speculation that C. hyalina may store sperm (Cancino, 1983), has been vindicated in the present study (see Chapter 3). The association of C. hyalina with an ephemeral substratum (Cancino, 1986), may make it advantageous to store sperm thereby assuring a temporal sperm supply and not relying on the next sperm input becoming available. Presence of progeny brooded in ovicells placed beyond the original colonial growing edge suggests that sperm movement within the colony, which might be expected if interzooidal connection throughout the funicular system, is present between contiguous zooids. Consequently, sperm could be transported from unhealthy to healthy areas within the colony to assure nutritional benefits provided by active autozooids beneath the ovicells.

9.4 Sperm precedence

The low values of P_2 recorded in the present study (see Chapter 6) are likely to be an adaptation to guarantee fertilization once sperm are available, so avoiding risk of egg wastage as a consequence of lack of future sperm supplies. This bias in sperm precedence favouring early sperm (i.e. first in / first out bias) could be explained by the first sperm being stored close to the anatomical place were fertilization takes place. However, the possible existence of physiological or biological sperm blockers, triggered by early sperm,

could also act against the latest sperm to arrive. By providing the opportunity to father the progeny using the first sperm pool, the present findings suggest that P_1 precedence may have evolved in C. hyalina as a mechanism to assure fertilization under restricted sperm supply. To date no information is available on sperm concentration from potential mates in colonies growing on their natural substratum. This information is important to understand fertilization dynamics in the field. Future studies should also include sperm suspensions of different genetic relatedness in order to test if the present pattern of sperm precedence is altered when receptor colonies acting as female colonies can alter the sperm usage.

9.5 Sperm competition and female choice

Despite the lack of only one sperm donor fathering all the progeny, i.e. maximum level of sperm competition or female choice, similar proportional fertilization by the 3 allosperm used in the cocktails can also be interpreted as sperm competition (see Chapter 7). Thus each allosperm donor was not allowed to father all the progeny. This can be interpreted as a consequence of receptor colonies using the available pool of sperm without discriminating among donors. Since sperm donor clones used in these experiments were a random population sample, they are presumed to be unrelated to the clones used as receptor colonies. Therefore, in the absence of receptor colonies being exposed to other levels of relatedness (i.e. full or half-sibs), the degree of mate choice among potential partners may be less important. Present studies in progress will answer the question if mate choice in C. hyalina is present when other levels of relatedness are involved.

9.6 Reprodutive allocation

The finding that colonies allocate more to reproduction once they are exposed to allosperm (see Chapters 4 and 7) is in agreement with findings of previous authors (Hunter and Hughes, 1983 c; Cancino et al., 1991). However, the present study is the first to confirm that contact between the receptor colonies and the sperm, and not a water borne effect associated with the sperm is responsible for triggering oocyte growing and budding of sexual zooids. These results are in agreement with similar findings in Diplosoma listerianum (JDD Bishop, personal comunication). This phenomenon allows energetic allocation into sexual processes only when egg and sperm come into contact inside the receptor colonies. Massive numbers of sexual zooids in colonies of C. hyalina growing in their natural substratum suggest that, despite sperm being exposed to turbulence causing

dilution and mechanical damage, they manage to reach receptor colonies. Gregarious settlement is maybe an adaptive mechanism evolved to cope with sperm dilution. Production of female zooids before males in small colonies under conditions of daily exposure to allosperm suspensions (see Chapter 7), strongly suggests that C. hyalina is not a protrandric. This is supported by observations of colonies growing in the field. Some colonies consisting of 20-30 basal autozooids bear complete and incipient female zooids and lack of male zooids.

9.7 Colonial fusion

Results on fusion in C. hyalina (see Chapter 8) are in agreement with other studies of bryozoans (Moyano, 1967; Chaney, 1983; Shapiro, 1992; Craig, 1994; Ishii and Saito, 1995). Moreover, the depenency of fusion on genetic relatedness suggests the existence of an allorecognition system, as has been found in other bryozoans (Chaney, 1983; Humphries, 1979) and has been proposed for many colonial marine invertebrates (Grosberg, 1988). The selective forces promoting fusion-nonfusion linked to allorecognition in C. hyalina could be related to gregarious settlement leading to contact between closely settled colonies. Lack of fusion among unrelated colonies could have evolved to avoid somatic and cell-germinal parasitism, phenomena well documented in the literature (Rinkevich and Weissman, 1992; Pancer et al., 1995). The present research recorded fusion among fragments of both the same genotype and full sib colonies and among colonies produced by sibling larvae. However, the presence of coalescent zooids was not recorded in colonies in contact that were growing on their natural substratum. This suggests that gregarious settlement in the field could directly result from larvae reaching available substrata as a function of environmental conditions (i.e. water flow regime), rather than behavioural habitat selection. Under this scenario, a mixture of larvae with different origins could be transported from the maternal colonies to the settlement area, resulting in random mix of unrelated genotypes.

On the other hand, fusion with maternal colonies and siblings could be a mechanism evolved to improve chances of survival as a chimeric organism. This has been demonstrated in chimeric demosponges, where chimeric sponges have higher chances of survival than non-chimeric sponges (Maldonado, 1998).

Appendix 1

Cloning and Rearing

Throughout this thesis numerous copies of each experimental clone (i.e. ramets) were needed. In order to carry out experiments under conditions of strict reproductive isolation, the method described by Hunter (1991) for propagating C. hyalina on glass proved inadequate. I therefore settled larvae onto pieces of conditioned acetate sheet, which is more easily cut onto small fragments. Below, I have described the techniques used to replicate founder experimental clones and to rear mass cultures.

A proper adhesive:

Small fragments of colonies produced by cutting founder colonies into pieces previously have been attached to the experimental substratum using silicon sealant (Hunter & Hughes, 1993a and b). However, personal observations suggest that silicon sealant has deleterious effects in early colony performance. Most of the experiments related to the objectives of my study involved the generation of numerous and healthy copies of the same genotype (i.e. clonal propagation). Accordingly, the main goal of this small trial experiment was to test if silicon sealant has deleterious effects and, if so, to find an alternative and harmless adhesive suitable to attach small fragments of C. hyalina colonies. Silicon sealant and an alternative adhesive, cyanoacrylate, were used as glues. The effect of the two different adhesives was examined using fragments of colonies originated from 2 founder colonies growing on acetate. The two founder colonies were established and grown using the protocol described in the colony bank (below). Once the colonies reached a large size, from each one six small sections (ca. 1.5 x 4 mm) were cut from the edge of each colony using the protocol described under cloning protocols (below). The resulting small pieces of acetate sheet bearing fragments of colonies were attached to a piece of acetate sheet (ca. 7.5 x 4.5 cm) using silicon sealant and cyanoacrylate as adhesives. Three small fragments were glued to one acetate sheet using silicon sealant and the remaining fragments to other acetate sheets using cyanoacrylate. Trios of small colonies originating from the same colony (i.e. clonemates or ramets) and attached to the substratum using the same glue were kept in the same vessel. To ensure that initial fragment size had no effect, the number of zooids on the small pieces of acetate were reduced to 10 using a needle to remove unwanted zooids. As a control to

the glue treatment the remaining zooids in the original colonies were reduced to three groups of 10 autozooids using a needle. The acetate sheets were placed in flat-bottomed glass vessels filled with 300 ml of 0.2 µl filter UV treated seawater (hereafter FSW). Water movement was provided using the oscillating paddle system described in the culture section (below). The colonies were grown for eight weeks at 18 °C with a daily food supply of 100,000 cell·ml^{-1} (Hunter and Hughes, 1993a) and water changed every second day. One month later, each colony was drawn and the number of zooids was counted using a stereo-microscope combined with a camera lucida. Percentages of active zooids per colony, the total area and the shape of the colony were used as criteria of colony performance. The index of circularity was calculated by dividing the perimeter, derived from an hypothetical circular colony of equal area to the experimental colony, by the measured colonial perimeter. The index therefore has a maximum value of 1 when the colony is perfectly circular. The number of basal males was also analyzed at the end of the experiment and used to assess effects of the adhesives on the colony performance. The results of this trial experiment are show in Table 1. When silicone was used, 20-30 times more basal males were produced and there were negative effects on both colony size and proportion of active zooids per colony. These results demonstrate that cyanocrylate is the better adhesive for the purpose of my experiments. As in the present trial, presence of basal males was later found to be a consequence of stress conditions under laboratory conditions, since they are rarely present in colonies growing naturally on the field (see Chapter 7).

Table 1: Effects of silicone sealant and cyanocrylate glue on C. hyalina performance. IC is an index of circularity (see text) and active zooids were computed as the ratio of basal zooids with functional lophophores (i.e. bearing guts filled with microalgae), to total number of basal autozooids in the experimental colonies. In the counting of basal zooids, total number of zooids was derived from the regression of number of basal zooids on colony size (see Appendix 3), and number of recycling zooids and basal males were counted directly from the colonies. Data are mean values (SD).

	Silicone	Cyanocrylate	Control
Initial area (mm^2)	3.12 (0.26)	3.10 (0.23)	3.21 (0.29)
Final area (mm^2)	25.96 (3.41)	32.59 (1.80)	32.31 (2.25)
Initial IC	0.75 (0.08)	0.73 (0.08)	0.74 (0.05)
Final IC	0.69 (0.05)	0.93 (0.04)	0.92 (0.03)
Initial active zooids (proportion)	1	1	1
Final active zooids (proportion)	0.71 (0.10)	0.88 (0.05)	0.85 (0.04)
Initial (basal males/ basal autozooids)	0	0	0
Final (basal males/ basal autozooids)	0.114 (0.039)	0.006 (0.006)	0.004 (0.005)

Establishment of the experimental clones:

Cultures of the model organism in the present thesis, Celleporella hyalina, were established from founder larvae released from a group of colonies growing on fronds of Fucus serratus and Laminaria saccharina. These macroalgae were collected in April 1996 (clones A_1 to Z_1) and October 1996 (clones A_2 to Z_2) during low spring tides from the Menai Strait at Beaumaris, Anglesey, U.K (53°16'N; 4°05'W) (Plate 1). Full sib and half sib clones were produced by collaboration with K. Hoare. These clones were produced by manipulated mating between clones established in April 1996.

Appendix 1

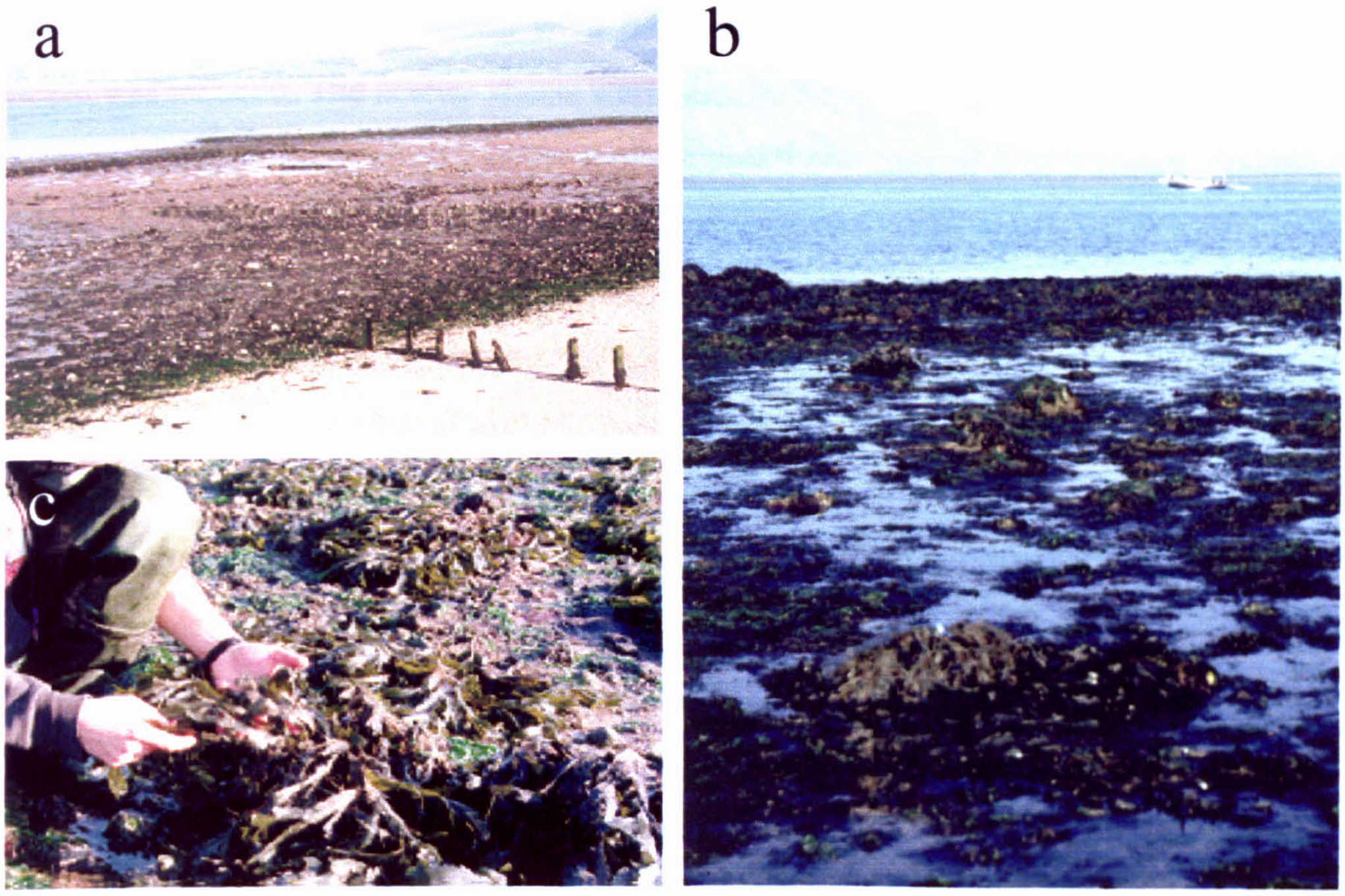


Plate 1: Location were experimental colonies of C. hyalina were collected in Beaumaris at the Menai Strait (a) landscape view of the collection place, (b) plants of Fucus serratus exposed to desiccation during a low spring tide and (c) fronds of F. serratus covered with colonies of C. hyalina.

After collection the samples were immediately stored in plastic bags and transported as soon as possible to the laboratory at Bangor. Upon the arrival to the culture room (Orton Building, School of Biological Sciences), fronds carrying colonies of C. hyalina were placed in a 40 l plastic tray filled with aerated filtered 0.2 μm and UV-treated sea water. Pieces of pre-conditioned acetate sheets (ca. 7.5 x 4.5 cm) were placed in the tray as a substratum for the larval settlement. Acetate sheets were considered to be pre-conditioned if they had been maintained in fresh water for a week and then placed in running seawater for a further week. Pre-conditioned sheets were used because settlement in bryozoans is positively related to the presence of biofilm in the surface of the substratum (Mihm et al., 1981; Brancato and Woollacott, 1982; Keough and Raimondi, 1995, 1996; Wieczorek and Todd, 1997). The tray was maintained in darkness for 24 h and larval release triggered by exposing the colonies to light (Ryland, 1960). Under laboratory conditions larvae of C. hyalina usually settle within a few hours of release from the parental colony (Orellana and Cancino, 1991, 1996; personal observations). However, to avoid damage of the primary zooids immediately after completion of

metamorphosis, the acetate sheets were removed from the tray one week after larval release was induced. The acetate sheets were removed from the tray and examined under the stereo microscope to select healthy-looking small colonies of 2 to 3 zooids. Healthy-looking colonies were considered to be those colonies with undamaged and active zooids, whose guts were filled with microalgae. Once the founder colonies were selected, the acetate sheets carrying the colonies were cut carefully with scissors and kept in different Petri dishes with FSW before being attached to new acetate sheets. To facilitate the handling of the founder colony, a narrow margin of acetate (ca. 2 mm) was kept beyond the growing edge of the colony (above). The selected colonies were attached to new pieces of acetate sheet (4.5 x 7.5 cm) using a pinpoint amount of cyanoacrylate (above) and grown on in isolation.

Most of the clones created using this procedure were maintained for the entire duration of the experimental program (April 1996-October 1999). Moreover, certain clones were adopted for several studies carried out by different researchers during this time and at the time of the writing are still under propagation.

Cloning protocols:

Cloned colonies can be created from a large parent colony of C. hyalina colonies growing on glass (Hunter and Hughes, 1991). However, inevitable wastage occurs when glass is cut to produce the desired number of colonial fragments (Hunter & Hughes, 1991; personal observation). In order to develop all the objectives of this dissertation it was necessary to produced a more efficient method for propagating from genetically unique founder colonies of C. hyalina. A suitable method of cloning is desirable because the number of colonies to be employed in the experiments is high. Colonies of M. isabelleana have been successfully cloned from colonies growing on pieces of acetate sheets (Manríquez, 1990). These colonies were attached to this substratum using protocols described by Cancino et al. (1991). Once they were big enough, the colonies were cut into small sections and glued onto the experimental substratum. In this dissertation I modified this protocol and I started from founder colonies settled under laboratory condition on acetates sheets (above). After the colonies reached a sufficient size to be cut, small pieces of acetate sheet bearing colony fragments (ca. 20-40 basal

zooids) were removed from the natural growing margin of the colonies (Plate 2). Only pieces of acetate bearing the most undamaged and healthy zooids were selected for attachment to the new substratum. The pieces of acetate sheet were trimmed from the founder colonies with scissors and the resulting pieces placed carefully in a Petri dish with FSW (Plate 2). In order to facilitate the handling of the colony fragments, a narrow margin of acetate (ca. 2 mm) was kept beyond the growing edge of the colony. Each small piece of acetate bearing a colony fragment was carefully glued to individual pre-conditioned acetate sheet (ca. 4.5 x 7.5 cm). Each small piece of acetate sheet was blotted using an absorbent tissue and placed on a pinpoint amount of cyanoacrylate located in the middle of the substratum (Plate 2). To avoid damage, the colony fragments were handled carefully from the acetate margin with the aid of thin tweezers. Any excess cyanoacrylate beyond the margin of the small piece of acetate sheet was immediately removed with absorbent tissue. In order to retain the founder colony, it also was glued to a new acetate sheet using the same method employed for the small fragments. Then, after drying (ca. 20-30 sec), each transplanted clonal fragment and the founder colony were placed in the culture system (above). A total of 12 colonial fragments (i.e. clonemates) and the founder colony were placed in each plastic histological rack inside the culture container (Plate 2). When more clonemates were produced, the extra colonies were kept in other racks (above).

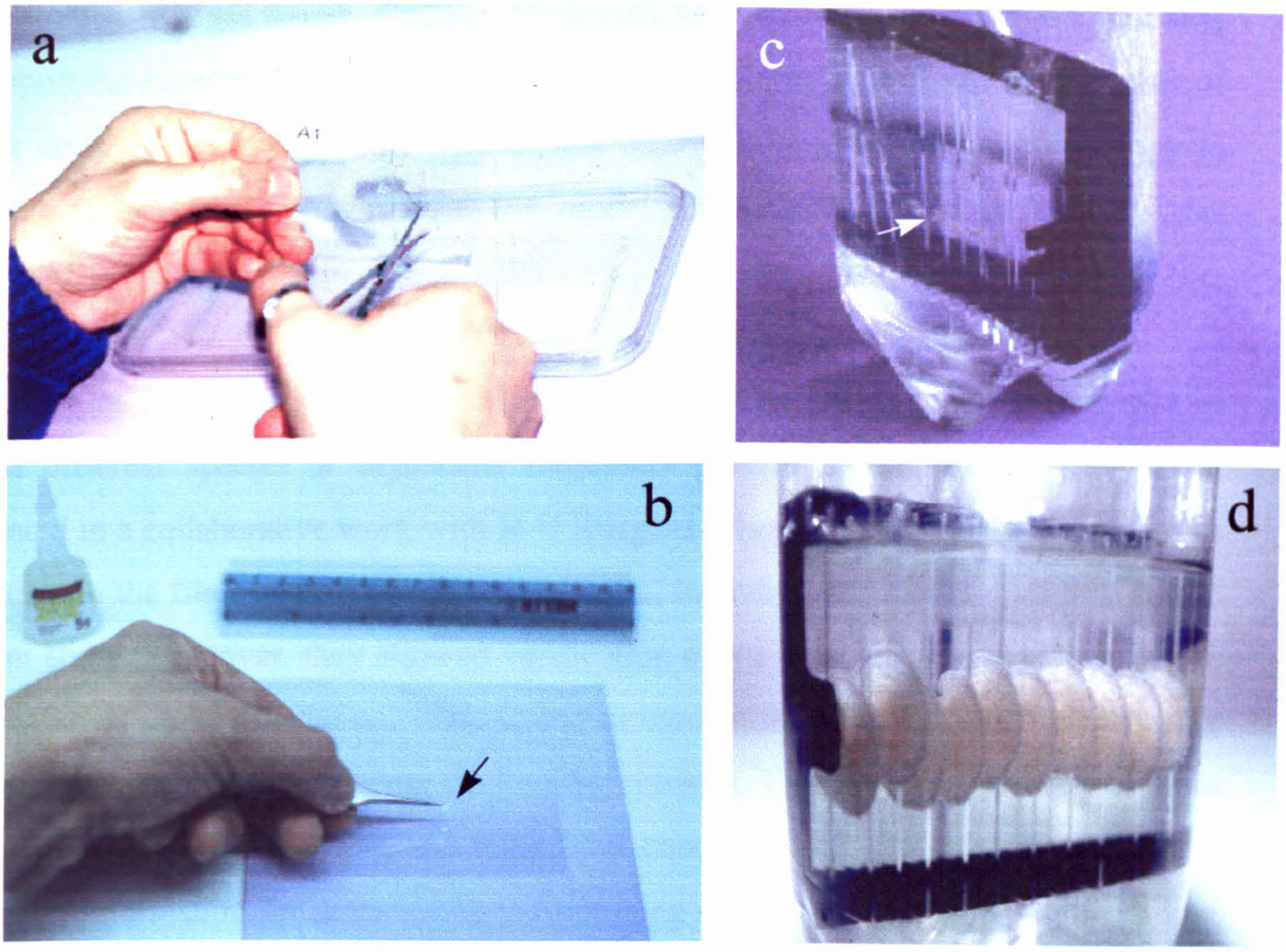


Plate 2: Cloning procedure (a) small sections of acetate bearing fragments of colonies are cut from a founder colony, (b) section are glued into the experimental substratum using cyanocrylate and then (c) placed in the corresponding rearing PET bottle holding in a histological rack until they reach the experimental size (d). The arrows indicate small fragments of colonies.

This method allowed me to obtain the larger number of copies required for all my experiments. Moreover, the success of this method is shown by its adoption in published (see Riisgård and Manríquez, 1997; Riisgård and Goldson, 1997) and unpublished studies with colonies of C. hyalina (Ruiz, 1998; Stuart, 1999).

Feeding:

Bryozoans are active suspension feeders creating feeding currents with their crown of tentacles. Previous laboratory work performed on C. hyalina has shown that maximum growth may be obtained by feeding colonies a concentration of 100,000 Rhinomonas reticulata cell·ml^{-1} (Hunter and Hughes, 1993a). The former work showed that although cell concentration influences somatic more than sexual parameters, an apparent trade-off between male production, female investment and food supply exists. The optimal food concentration found by Hunter & Hughes (1993a) exceeds several times the

concentration of algae found in nature. Moreover, this algal concentration was not able to support the expected high percentages of active zooids during the culture of the set of clones during April 1996. In the light of this information, a different methodology to assess a proper feeding regime for the model organism in the present study was desirable.

The study of particle capture mechanisms and quantification of the pumping rate in fifteen different species of bryozoans frequently present in the Menai Bridge was evaluated in a collaborative work with H.U Riisgård. These parameters are important to characterize the filter-pump of marine bryozoans, including the model organism in the present study. Moreover, they allowed verification of the food supply necessary for the most efficient culture of the colonies. Colonies used in this study were obtained using the same protocols used to procure the founder colonies. The clearance capacity of these colonies was evaluated at low and high food concentrations. In order to obtain the necessary information for determining the pumping and clearance rate, video recording of food particles close to the lophophores (Fig. 1) and an electronic particle counter were utilized.

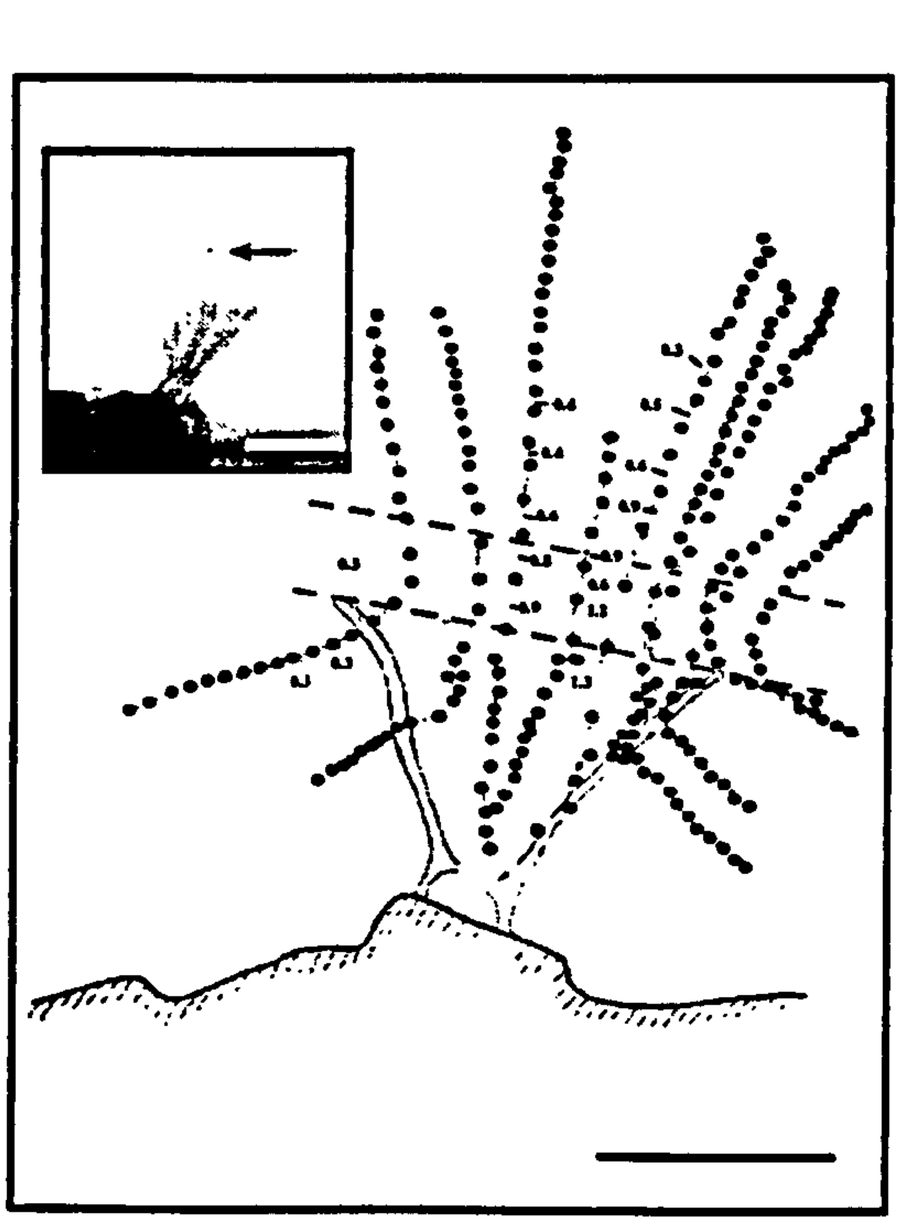


Figure 1: Drawings of food particle trajectories close to a protruded C. hyalina lophophore derived from video recording (insert picture). Scale bars = 200 µm.

Appendix 1

A full description of the methods and analyses used in the filter-feeding study are beyond this thesis, but can be inspected in the paper produced in the former study (Riisgård and Manríquez. 1997). Food concentration (100-200 cells.μl^{-1}) suggested in this study were successfully used in the rearing of my experimental colonies These concentrations of food, able to support colonies of C. hyalina, was corroborated and published in a different study as optimal food concentration for C. hyalina (Riisgård and Goldson, 1997).

Massive and single rearing systems:

Several culturing methods of bryozoans have been reviewed by Jebram (1977 and 1980). Colonies of C. hyalina have been cultured in the laboratory using several protocols (e.g. Cancino 1983; Hunter and Hughes 1993a, b and c; Goldson, 1999). However, most of the conventional bryozoan culture methods include air bubbling. Under these conditions, chances of cross contamination are extremely high given the high densities of colonies and the contiguous culture used in the present study. It was thus necessary to develop an effective method for culturing clones of bryozoans under reproductive isolation was required to develop. Chances of sperm contamination through air-borne spray between different clones was overcome by using three alternative culture systems. Moreover, special precautions to minimize contamination were considered in the manipulation of the colonies. Below I describe the three culture systems that have been implemented in order to rear the model organism under laboratory conditions

a. Oscillating paddle system:

In this system individual colonies were maintained separately in 300 ml flat-bottomed glass vessels or alternatively in groups of 12 clonemates in 800 ml plastic boxes. Water movement was provided by Perspex paddles. Four racks of oscillating paddles with room to keep simultaneously 40 vessels or 20 plastic boxes were assembled during the beginning of my experiments (March-April 1996). In the vessels, the colonies growing on acetate sheets were placed vertically, touching the paddles. In plastic boxes, the colonies were placed in histological staining racks using glass slides (ca. 7.5 x 4.5 cm) to keep the acetate sheets in place. Movement of the paddles was generated using an

electric motor and a specially designed apparatus where the paddles were pushed actively forward and then passively returned to the original position. Thus the forward and backward displacement of the paddles originated a continuous and gentle movement of the water contained in the vessels or in the plastic boxes (Fig. 2 and Plate 3). No deformation of the acetates bearing the colonies was produced as consequence of the water movement.

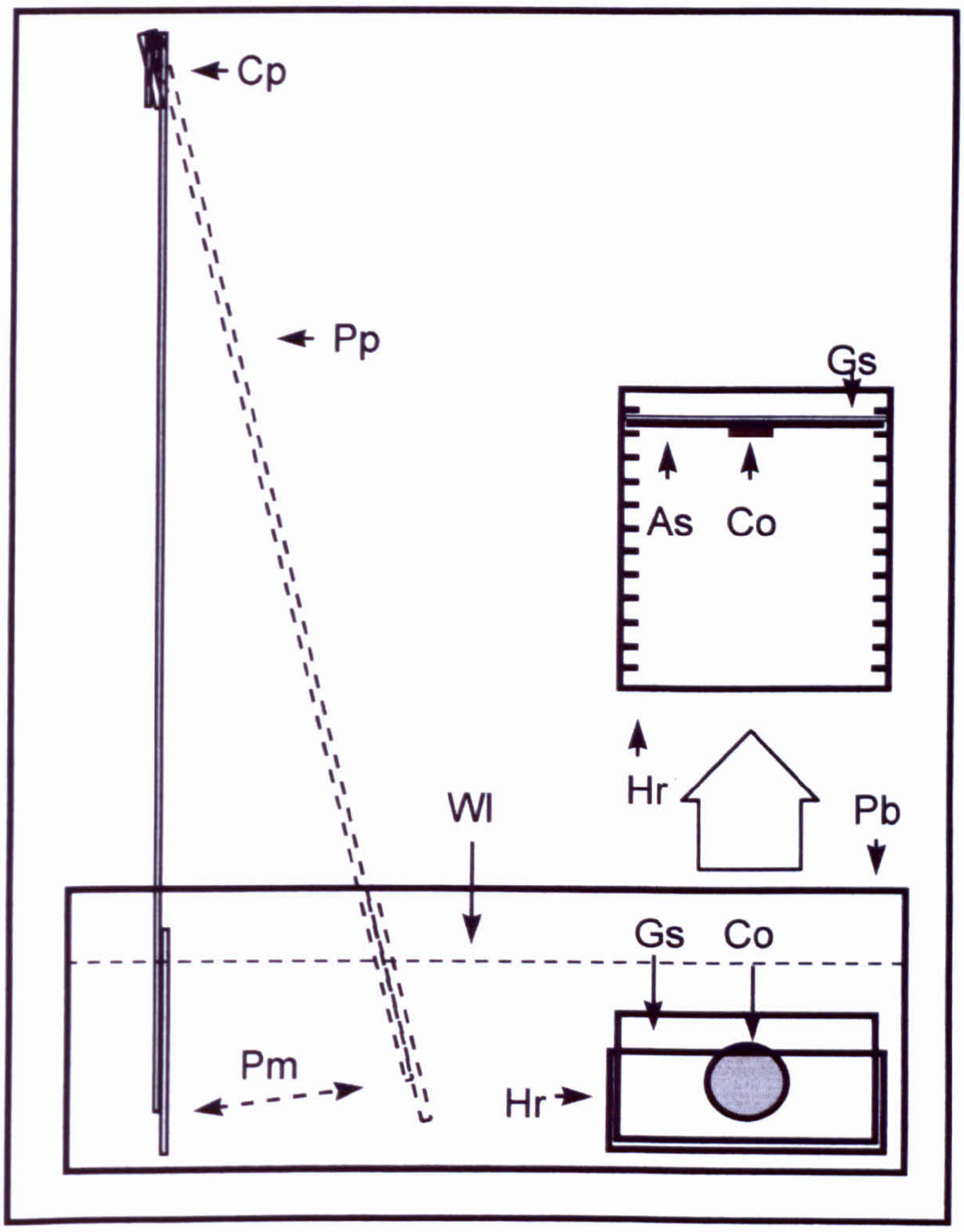


Figure 2: Schematic (not to scale) representation of the oscillating paddle system used for rearing clone mates of C. hyalina in the laboratory under reproductive isolation. As, acetate sheet bearing a colony; Co, colony of C. hyalina; Cp, centre of pivot; Gs, glass slide; Hr, plastic histological rack; Pb, 800 ml plastic box; Pm, movement of the paddles; Pp, Perspex paddle; Wl, sea water level.

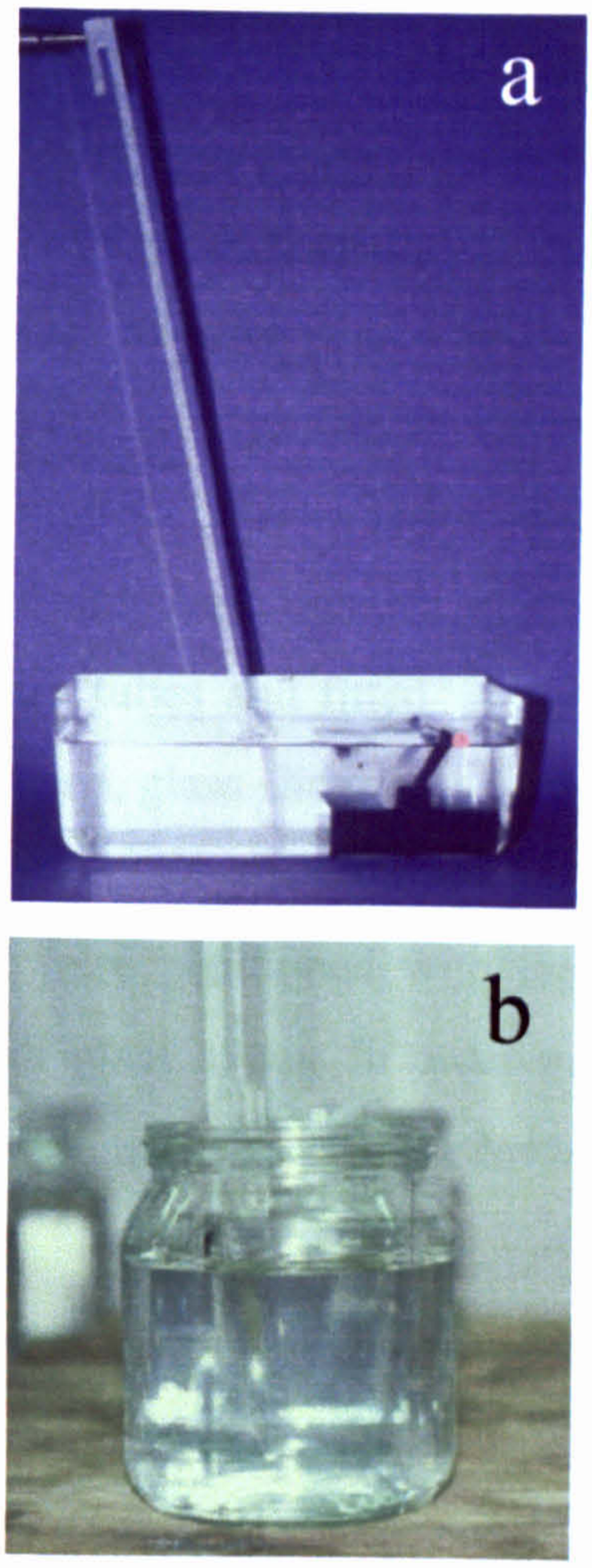


Plate 3: (a) oscillating paddle system operating in a plastic container bearing ramets of C. hyalina and (b) oscillating paddle system operating in a 300 ml flat-bottomed vessel bearing a single colony of C. hyalina.

b. Aerated system 1 (PET bottles):

Clear polyethylene terephthalate (PET), cylindrical bottles with a maximum capacity of 2 l have previously been used in conjunction with histological racks to rear colonies of C. hyalina (Goldson, 1998). The PET bottles are strong, lightweight, cheap and, as they are used to make bottles for water, soft drinks, juice, liquor, and numerous other food and non-food items they are not toxic. In order to improve PET bottles as a rearing system for my experiments, modification were made to the top of each bottle opening. Clear covers consisting of plastic dishes with two perforations were placed on the top of each bottle. A manually operated air valve mounted in the central perforation of the cover was used to control the input of air through a stone air releaser placed in the bottom of the bottles. Flexible plastic airline tubing was used. An air vent was mounted in the second perforation, consisting of a silicon tube connecting the interior and exterior of the bottles. To avoid any possible spray contamination, plastic pipettes with a perforated bulb were joined to the external side of the silicon tubes and fitted with a wool filter in the bulb (Fig. 3). When histological racks were used, glass slides (ca. 7.5 x 4.5 cm) were used to keep the acetate sheets in place (Plate 4). The glass slides were distanced every 0.5 cm in the rack and the acetate sheets were positioned with the fragments of colonies facing the space between two adjacent glass slides. In order to avoid accumulation of waste products on the surface of the colonies, the acetate sheets were vertically placed in the containers (Fig. 3 and Plate 4).

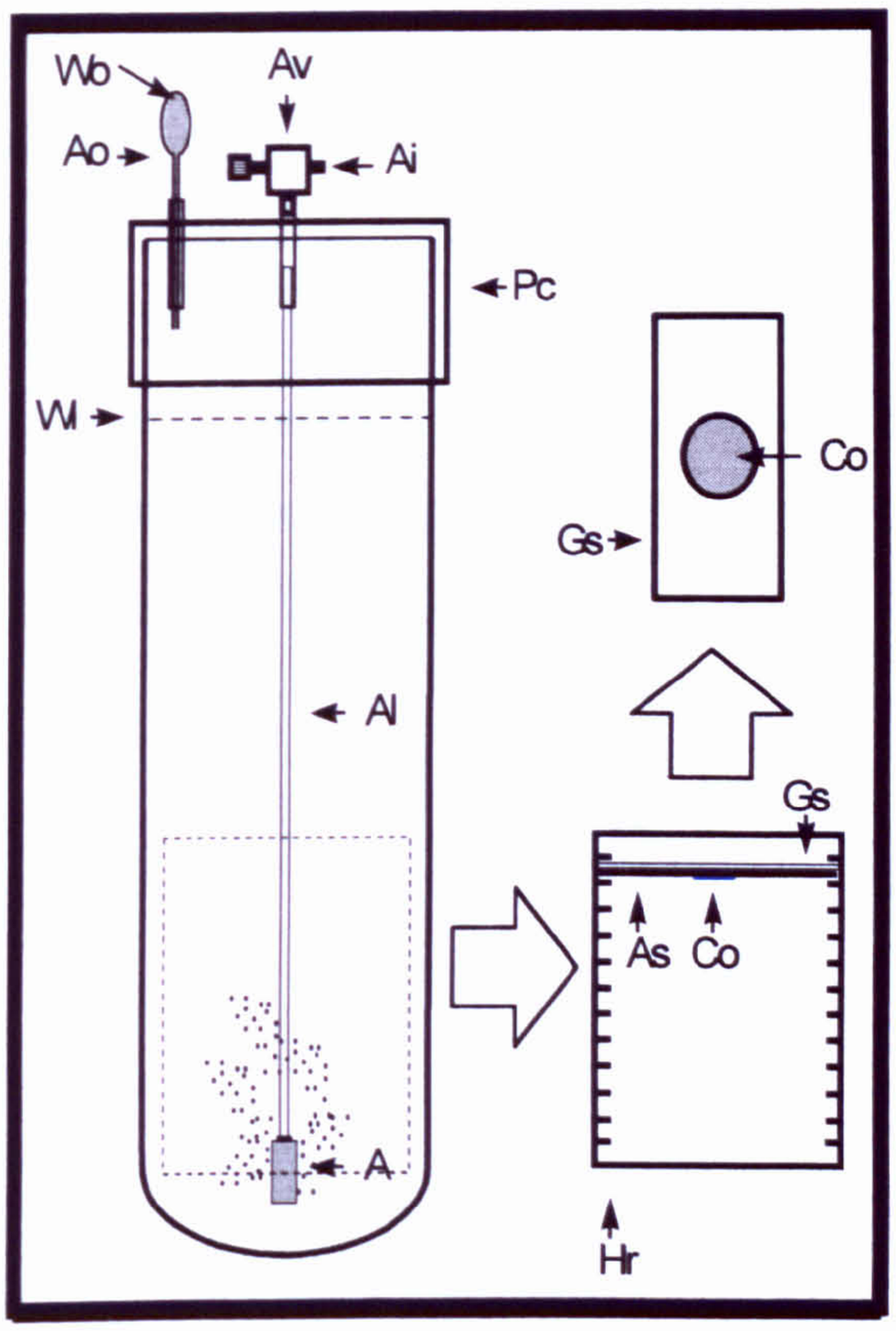


Figure 3: Schematic (not to scale) representation of the experimental 2l (PET) bottles used for rearing clone mates of C. hyalina in the laboratory under reproductive isolation. A; air stone releaser; Ai, air input; Al, plastic air line; Ao, air ouput; As, acetate sheet bearing a colony; Gs, glass slide; Co, colony of C. hyalina; Pc, plastic cover; Wo wool filter.

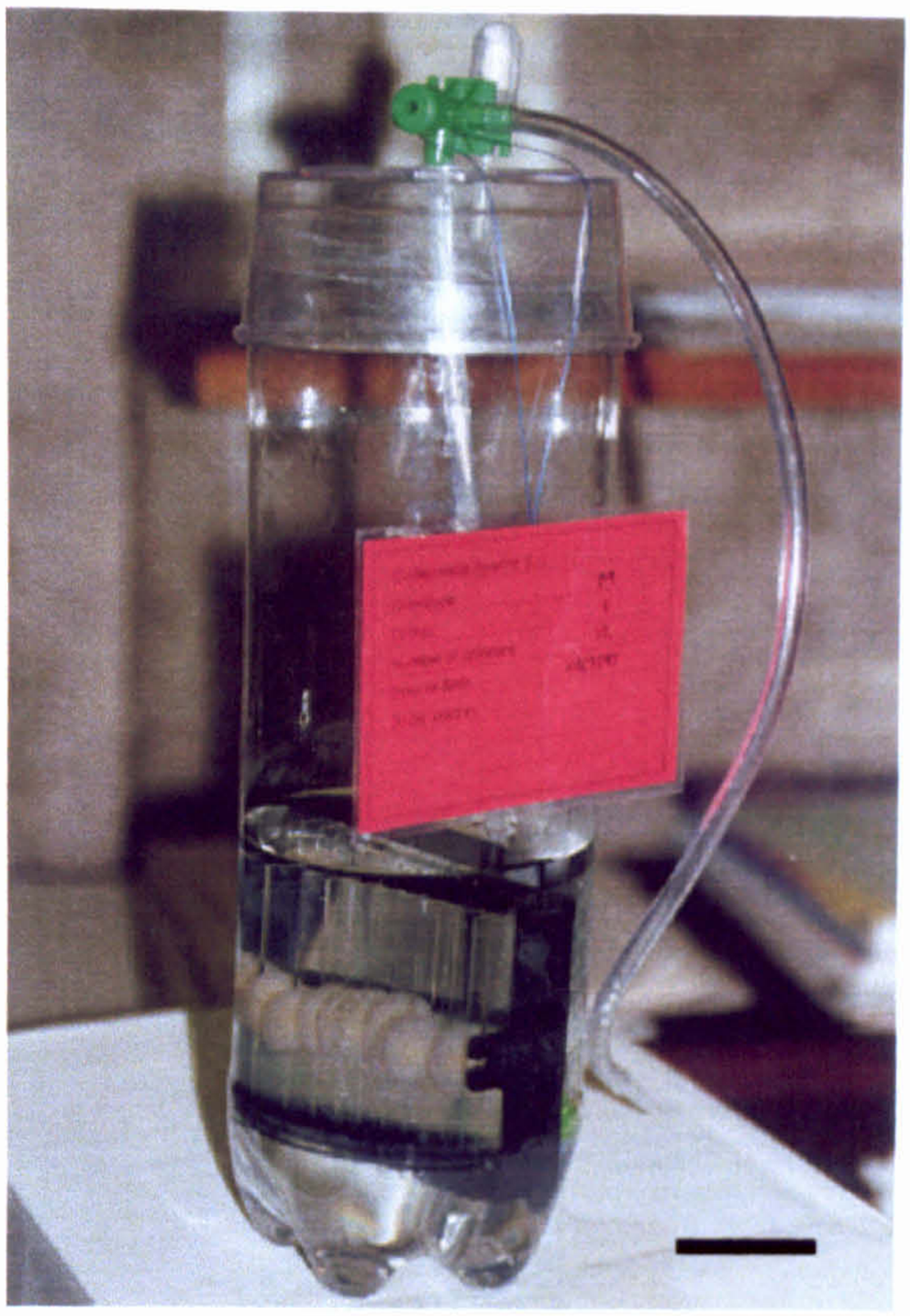
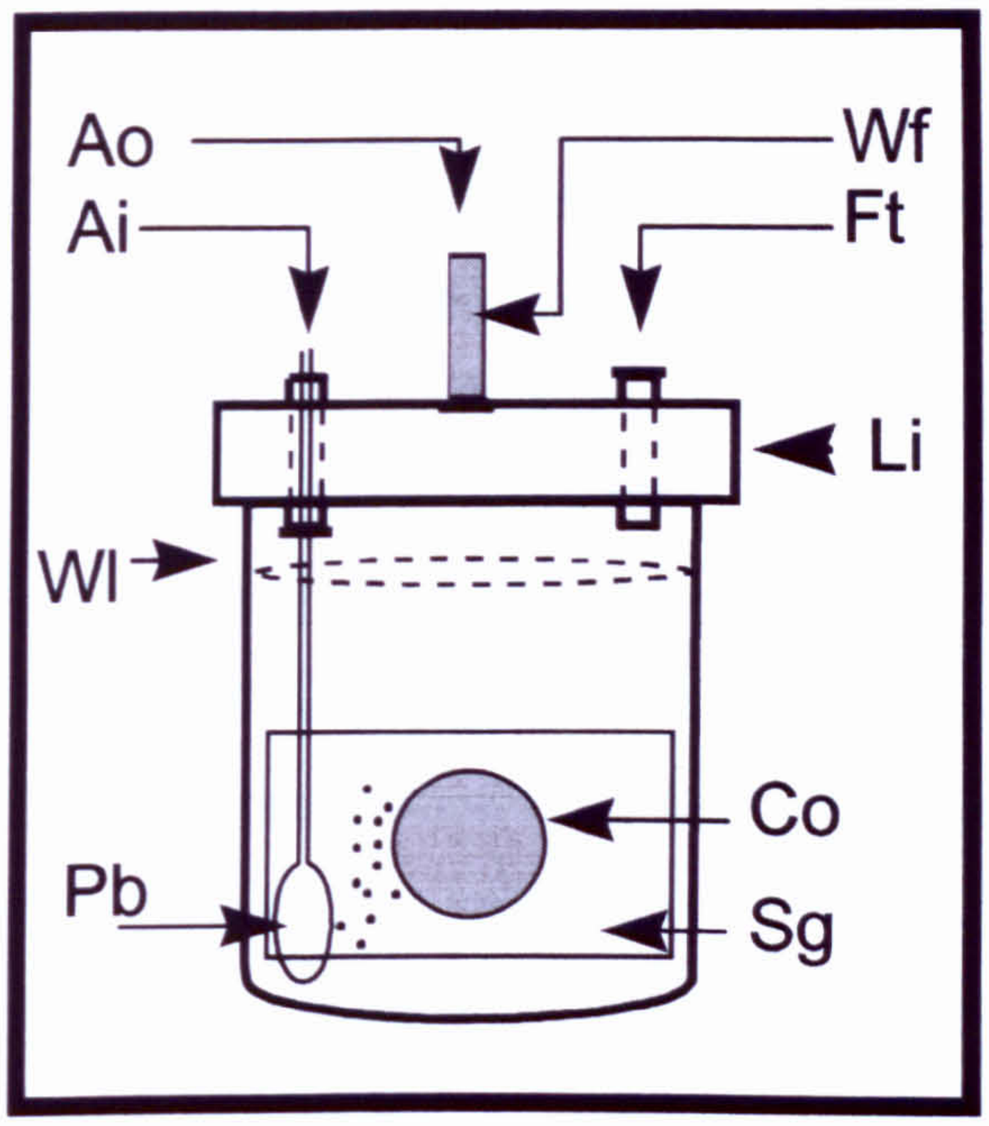


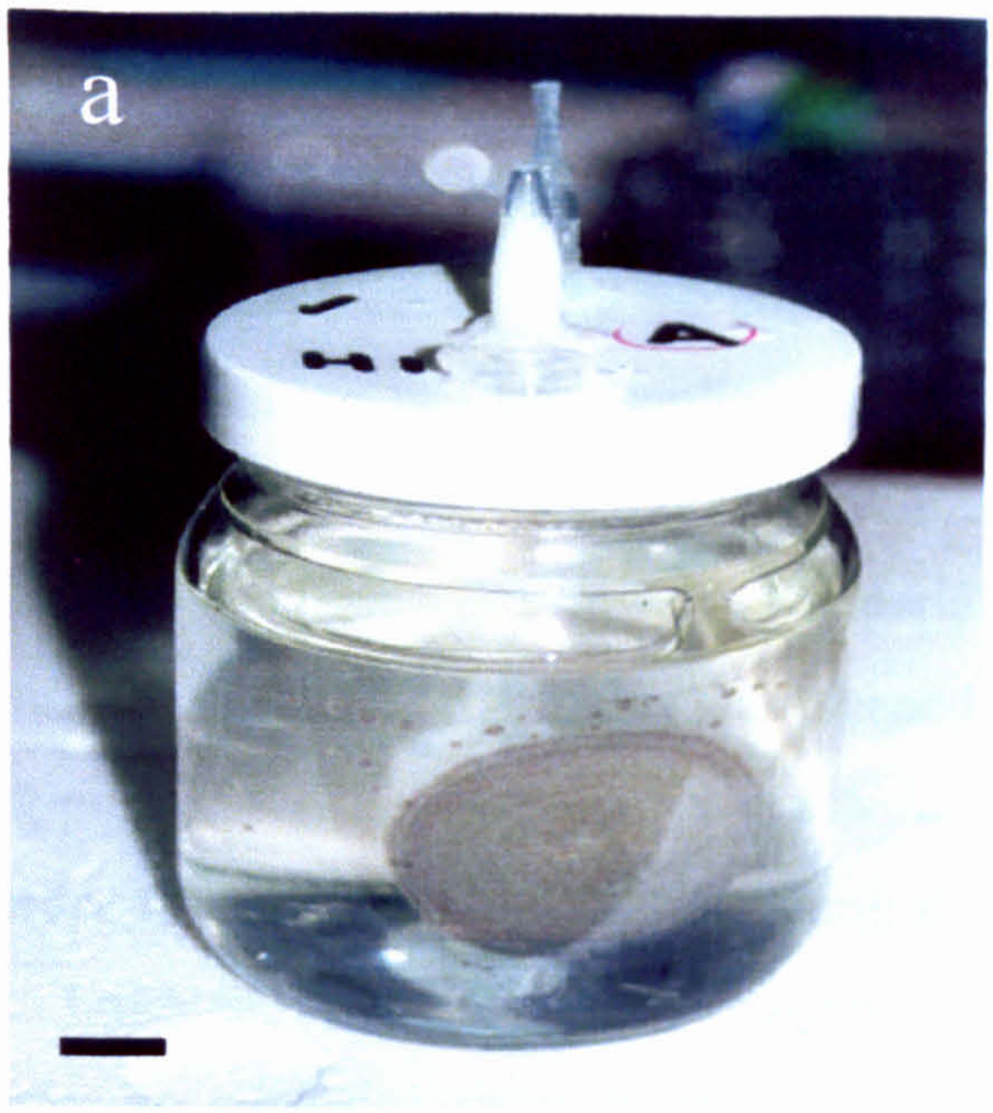
Plate 4: PET bottle with a histological staining racks bearing 12 colonies of C. hyalina. Red card hanging from the bottle's neck summarizes information about the colonies such as: clone, number of colonies, date of cloning and experiments where the colonies will be used. Scale bar = 4 cm.

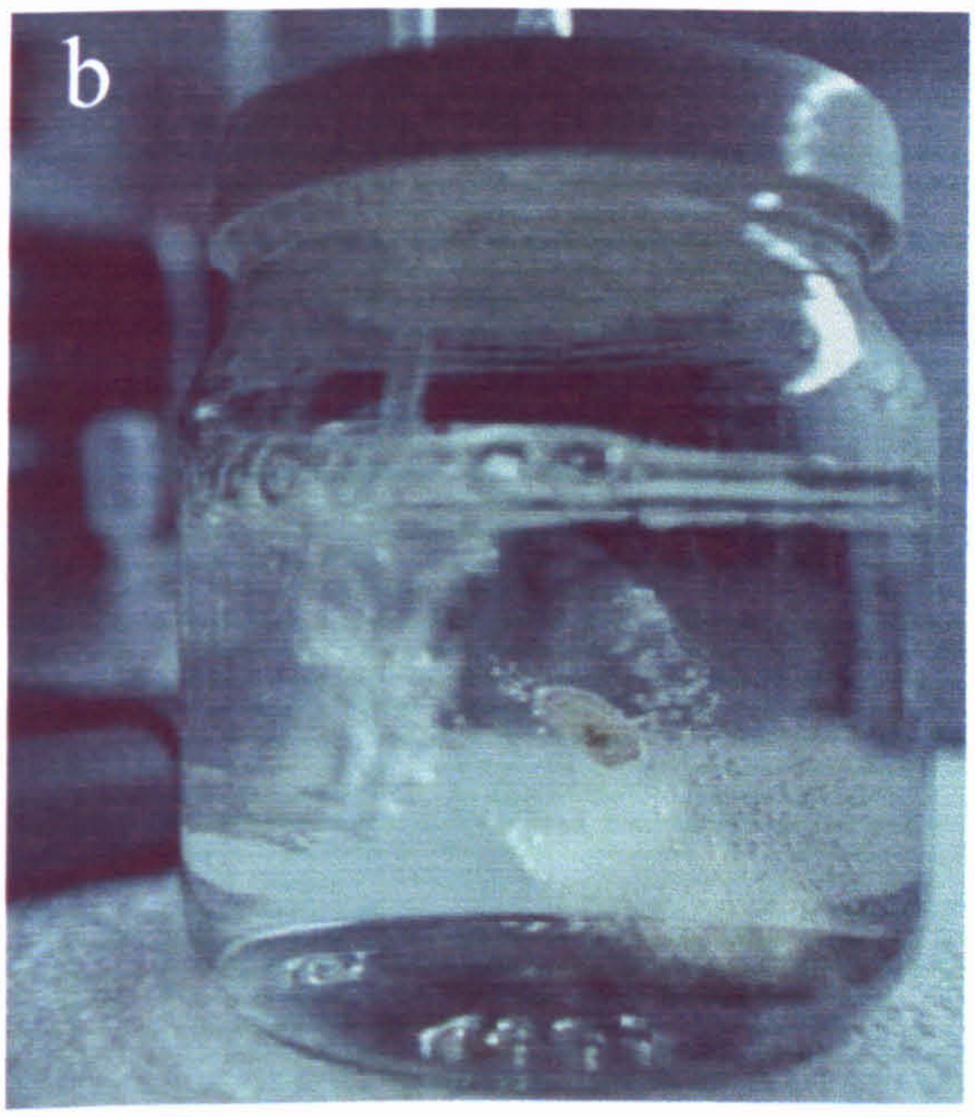
c. Aerated system 2 (Flat-bottomed glass vessels):

The paddle system was not successful in the new laboratories at Brambell Building (School of Biological Sciences) as a consequence of the prolific growth of bacteria using plasticizers dissolved from the air into the water, as a substrate. A second emergency culture system was developed and implemented from February of 1997. This new system was designed in order to maintain in reproductive isolation single colonies. In this system the colonies were maintained separately in 300 ml flat-bottomed glass vessels closed with a perforated plastic lid (Fig. 4 and Plate 5a). An Eppendorf micro-tube was mounted in each of three holes through the lid, using melted plastic. One micro-tube from which the apex and lid had been removed, carried a plastic pipette

connected to an air supply that had passed across activated charcoal and then through distilled water to remove potential contaminants. Small perforations in the bulb, which was placed close to the bottom of the vessel, facilitated the desired amount of bubbling (Plate 5b). Air was vented through small perforations made in the apex of the second micro-tube, protruding vertically from the centre of the vessel's lid. The third micro-tube, with apex removed but retaining its own lid, served as a port for injecting algal suspension (Plate 5c).







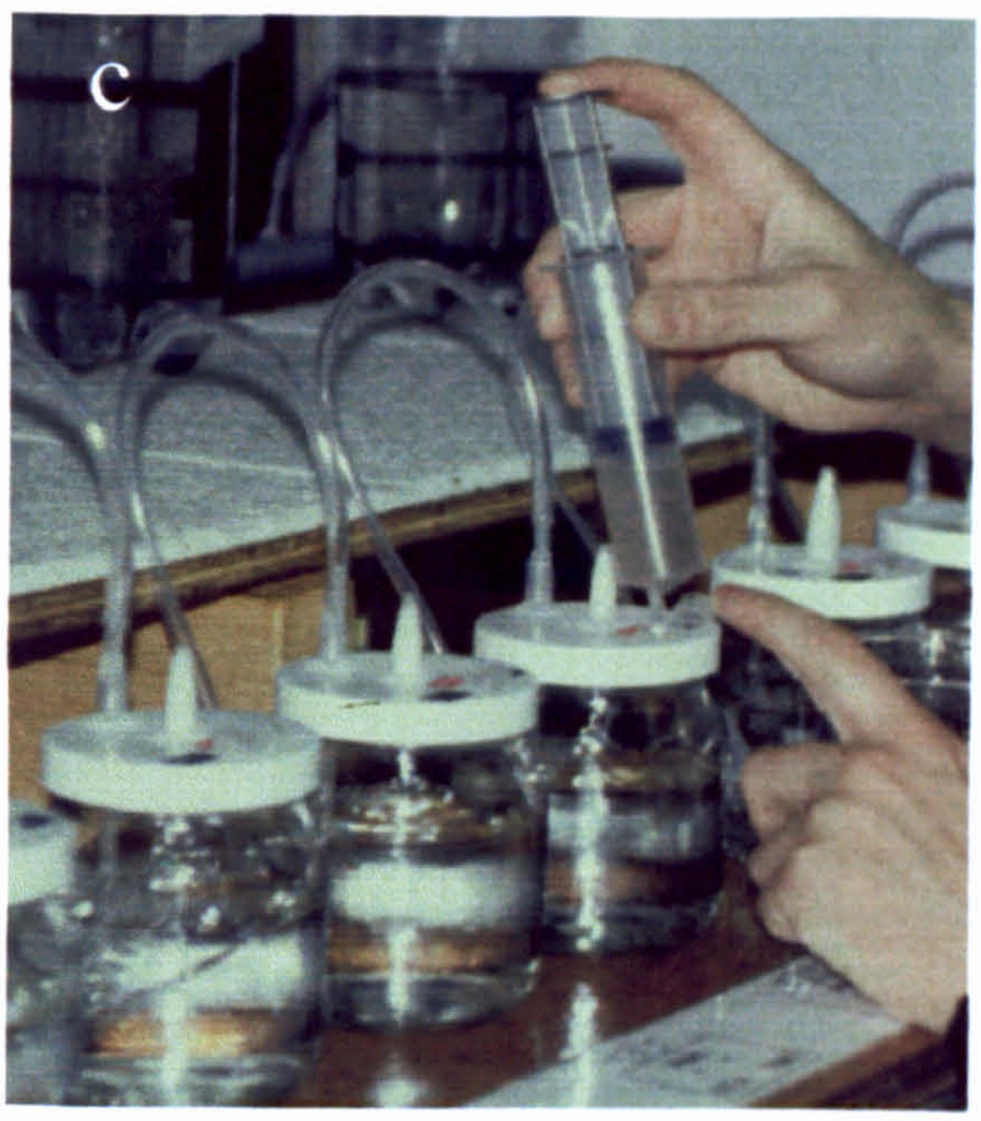


Figure 4: Schematic (not to scale) representation of the 300 ml flat-bottomed glass vessels used to rear in reproductive isolation single colonies of C. hyalina. Ai, air input micro tube; Ao, air output microtube; Wl; water level; Pb, plastic pipette with perforations; Sg, glass slide; Co, colony of C. hyalina; Li, perforated plastic lid.

Plate 5: (a) 300 ml flat-bottomed glass vessel with an experimental colony of C. hyalina inside, (b) Bubbles leaving the perforation in the pipette producing aeration and the water movement needed to maintain food cells in suspension and (c) microalgal food being supplied into the experimental vessels through the aperture in the plastic cover of the vessels. Scale bar = 1 cm.

Cleaning methods:

Colonies

Depending on the experiment the frequency of cleaning varied. However, the colonies were always cleaned using the same method. The acetate sheet bearing the colony was placed over the surface of a clean, white piece of plastic. In this way, fouling material growing on the surface of the acetate was easily revealed. Thus, with the aid of a soft artist's brush the surface of the acetate was swept to remove the unwanted material (Plate 6). The same procedure was repeated when the surface of the colony was cleaned. Care was taken when the face of the acetate opposite to which the colony was attached was cleaned. This precaution was taken in order to avoid pressing and damaging the colony against the white plastic. In addition to brushing, FSW was gently sprayed into the surface of the colonies (Plate 6). In order to minimize stress to the colonies, throughout these proceedings colonies were maintained most of the time under FSW.

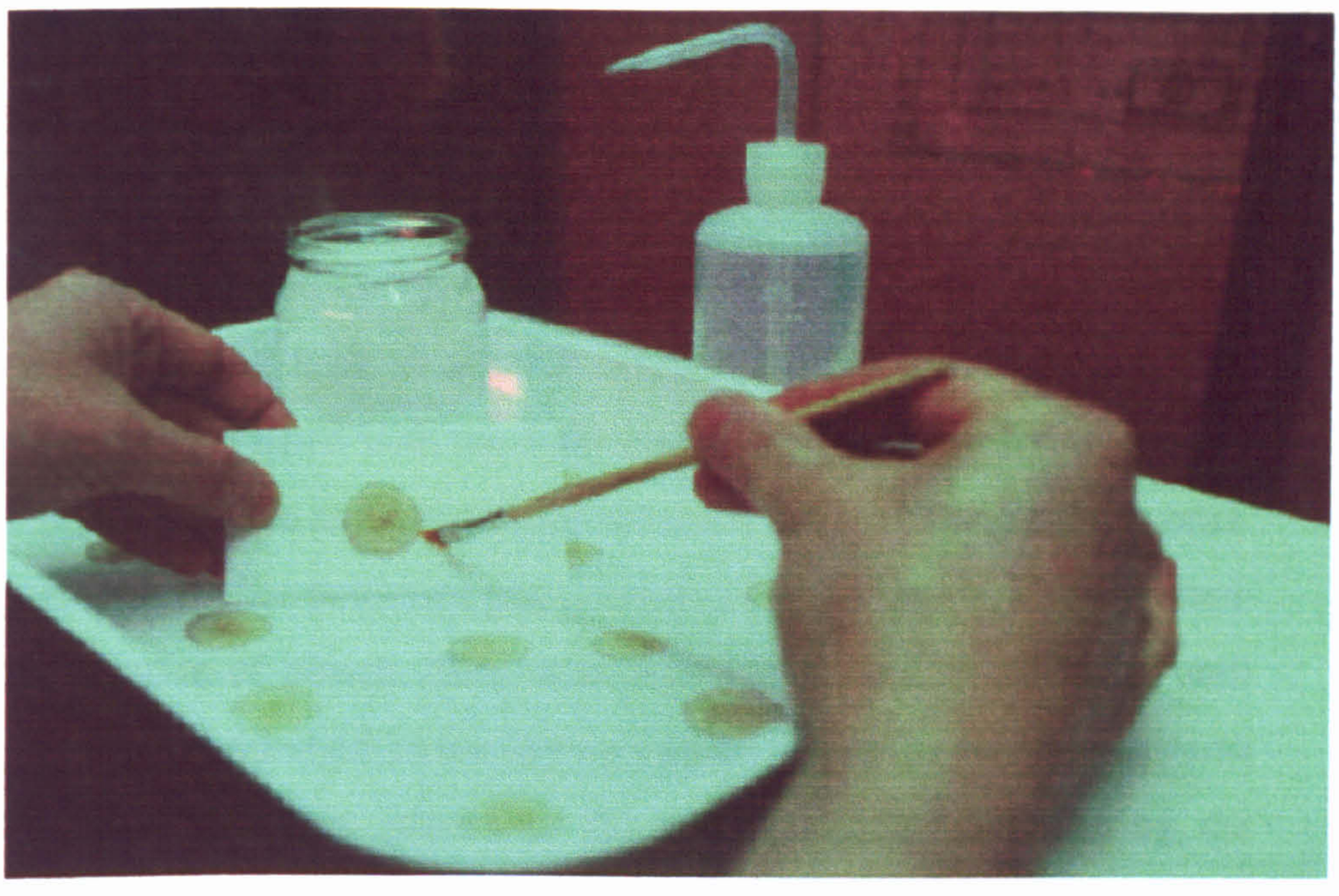


Plate 6: Cleaning procedure. Colonies are placed on top of a piece of white plastic and with the aid of a soft artist's brush the surface of the colony and the acetate are cleaned and then sprayed with FSW.

Histological staining racks and glass slides

In the mass rearing, once a week the histological staining racks were cleaned with hot tap water. With the aid of a modified toothbrush, all the surfaces of the racks were brushed and fouling material removed. The glass slides used to hold the acetate sheet bearing the experimental colonies were also cleaned weekly with hot tap water, using the same tooth brush as above. Once a month, all the glass slides in the racks were replaced with clean ones. The old slides were maintained overnight in a weak solution of HCl or bleach and then keep in running tap water. Histological racks and glass slides were dried off in a dry cabinet before being used again.

PET bottles and Flat-bottomed glass vessels

PET bottles were cleaned once a week using tap water and a soft sponge. Care was taken in order to avoid scratching the inner walls of the bottles. Flat-bottomed glass vessels were cleaned at a frequency depending on the experiment, but always using tap water and the drying cabinet to dry them off.

Aeration systems

The aeration system in the PET bottles was cleaned once a week using hot tap water. Air stones were cleaned by keeping them in a weak solution of HCl or bleach and used again only after being totally washed with tap water and dried off in the drying cabinet. Every third month, both the airline and the airstone releasers were replaced by new ones. The aeration systems from the flat-bottomed glass vessels were cleaned by keeping them overnight in a weak solution of bleach. They were then maintained in tap water until totally free of any bleach and dried off in the drying cabinet. Once the aeration systems were dried, a new wool filter was placed in the corresponding tube. During the experiments, the wool filter was replaced weekly. When flat-bottomed vessels were used, two aeration systems per vessel were installed. When one system was removed for cleaning, there was always a second one clean ready to be used.

Appendix 2
Sperm release, sperm counting and the effect of sperm concentration on fertilization success

Manipulations of sperm suspension were required in most of the experiments in the present thesis. Moreover, in order to avoid problems associated with sperm age and sperm concentration confounding experimental results an appropriated method was needed to count fresh allosperm suspensions and evaluate possible consequences of sperm concentration on fertilization success.

Sperm release

In bryozoans such as C. hyalina, sperm leave the male zooids through protruded male tentacles (Marcus, 1938, Silén, 1966, 1972). Light-triggered sperm release has been found in Ciona intestinalis (Woollacott, 1979). Moreover, dawn release of gametes has been demonstrated in the colonial hydrozoan Hydractinia echinata (Yund, 1990) and in seaweds (Clifton, 1997). However, to date no information is available about the control of sperm release in bryozoans. A trial experiment carried out during 1996 at the Marine Biological Association of the UK, The Laboratory, Citadel Hill, Plymouth, and subsequent experiments at Bangor allowed the potential role of light in triggering sperm release in C. hyalina to be investigated.

a. Plymouth trial experiment

This trial experiment was carried on during January 1997 using two sexually mature colonies of similar size (ca. 2 cm in diameter each). One colony was maintained in total darkness for 12 h and the other in continuous illumination for the same period of time. Colonies were kept in individual plastic boxes filled with still FSW at 18°C and fed with 1 ml of R. reticulata. After 12 h of treatment, both colonies were removed from the boxes, cleaned for 3 min with FSW and placed in clean boxes filled with FSW with water movement provided by an oscillating paddle system. Colonies were exposed to light for 15 min and then water samples of 30 ml collected and sperm counted (below).

The results of this trial experiment are shown in the Table 1. Approximately 70 times more sperm were found in the treatment dark/light than in the other treatment where the

colony was continuously exposed to light.

Table 1: C. hyalina mean number of sperm densities (sperm/ml) obtained in a colony dark-light treatment and in a colony continuously exposure to light. Data are mean ± SE, and * =p<0.0001 using the Mann-Whitney *U* test.

	continuous exposure to light	dark-light treatment	*U*
(sperm/ml)	0.83 (0.40)	57.83 (11.44)	*

b. Bangor experiments

Further experiments were conducted in order to verify the preliminary findings that light could trigger sperm release in C. hyalina. These experiments were similar to that previously described. However, two ramets of similar size from each of 4 clones were used. The experimental colonies were maintained in darkness for 12 h and then exposed to light for 15 minutes. However, the control colonies were maintained in darkness for 12 h and then changed to a new container with clean FSW kept in darkness. To maintain the water temperature at 15-16 °C two precautions were taken; the light was maintained 2 m distant from where the vessels stood in a water bath set at the experimental temperature. In order to avoid exposure to light, the water samples were removed by siphoning through a plastic airline connected to a syringe using the aperture for algal injection (see Appendix 1). All the water samples were placed in plastic containers and the sperm counted (above). Sperm counts were standarized by ml and number of males per colony.

The results of these experiments are summarized in the Table 2. Between 30 and 200 times more sperm were found in colonies maintained in the dark-light treatment than in the colonies maintained in darkness. Based on the results of the Plymouth and Bangor experiments (Table 1 and Table 2), I conclude that dark-light reaction (above) is a good method for inducing sperm release in C. hyalina. The light-induced sperm release was found in experiments conducted at different times during the day. This suggests that sperm release is not dependent on a biological rhythm and so can be experimentally manipulated at any desired time.

Table 2: C. hyalina mean sperm density (sperm/ml) obtained in colonies maintained in darkness and in colonies exposed to light after 24 h in darkness. Data are mean ± SD,

and * =p<0.0001 using the Mann-Whitney *U* test.

	Colonies exposed to light	colonies in darkness	*U*
clone 1 (sperm/ml/male)	14.63 (6.29)	0.07 (0.08)	*
clone 2 (sperm/ml/male)	8.98 (7.47)	0.05 (0.06)	*
clone 3 (sperm/ml/male)	2.29 (1.88)	0.07 (0.12)	*
clone 4 (sperm/ml/male	8.27 (5.53)	0.24 (0.10)	*

Contact time and sperm concentration

Two experiments were conducted to find optimal conditions for fertilization, prior to the main experiments of the present thesis.

a. Contact time:

The first experiment investigated the minimum time for which recipients must be exposed to allosperm in order to achieve fertilization. For each recipient clone, E_1, F_1, J_1 and Q_1, 14 ramets were placed in a plastic slide rack. Three beakers were filled with 1.5 l of compatible allosperm suspension from donor clone A_1. Once the allosperm suspensions were obtained (above), each rack bearing the recipient ramets was immersed in the allosperm suspension and one ramet of each clone was removed from the suspension at predetermined time intervals as follows: 1 min, 5 min, 15 min, 30 min, 45 min, 1 h, 1.5 h, 2h, 4h, 8 h, 10 h, 12 h, 24 h, 36 h and 48 h. A magnetic stirrer and water bath were used to minimize allosperm sedimentation while maintaining constant temperature. Sperm concentrations at the beginning of the exposures were over $1x10^{-3}$ sperm/µl. A few ml of R. reticulata were added to the beaker in order to enhance feeding activity. Once ramets were removed from the allosperm suspension they were rinsed with FSW for 5 min and assigned to individual experimental vessels. One ramet of each recipient clone was kept isolated from allosperm, serving as a control for self fertilization. After 1 wk, all ramets of each recipient clone were replaced in a slide rack and maintained in standard culture conditions (below) in 2 l PET bottles. A code was scratched onto the acetate bearing the colonies to identify the time each ramet was exposed to allosperm suspensions. Colonies were maintained in standard culture conditions (see Appendix 1) and number of embryos counted every 3 days. Fertilization success was calculated by dividing the number of

embryos originated during 2 months by the number of active zooids present in the colonies at the beginning of the experiment.

b. Sperm concentration:

The second experiment investigated the minimum concentration to achieve fertilization. For each recipient clone: A_1, D_1, E_1, H_1, J_1, M_1 and Q_1. Five virgin ramets were exposed to allosperm suspensions of the sexually competent allosperm F_1. Allosperm from the donor clone were obtained using the dark-light reaction (above) using 36 sexually mature colonies. From the initial allosperm suspension, 4 extra allosperm suspensions were obtained by diluting with FSW. These new allosperm suspensions were 10, 100, 1000 and 10000 times less concentrated than the initial suspension. Receptor colonies were individually exposed to 200 ml of allosperm suspensions in 300 ml vessels for 2 h, a few drops of R. reticulata being added to the vessel in order to enhance feeding activity. Once the colonies were exposed to allosperm suspensions, all ramets of each recipient clone were placed in a slide rack and maintained in standard culture conditions (below) in 2 l PET bottles. As a control for self fertilization, one extra ramet of each clone was not exposed to allosperm suspensions and placed in the same rack with the others. Fertilization success was assessed as in the previous experiment.

The effect of contact time between receptor colonies and the allosperm suspensions using non-limiting sperm concentration are shown in the Figure 1. Highest, similar and asymptotic values of fertilization success were obtained when the receptor colonies had been exposed to allosperm suspensions for more than 1h (Fig. 1). After the same period of exposure, embryos were not observed in the control colonies.

Although relatively few vitellogenic oocytes and embryos developed in ramets exposed to allosperm for 1-5 min, the number increased greatly for mating times exceeding 30 min, but reached asymptote at 60-120 min (Fig. 1). Subsequent experiments therefore used mating times of at least 60 min. As in the previous experiment, after the same exposure time embryos were not observed in the control colonies. The effects of sperm concentration on fertilization success are shown in the Figure 2.

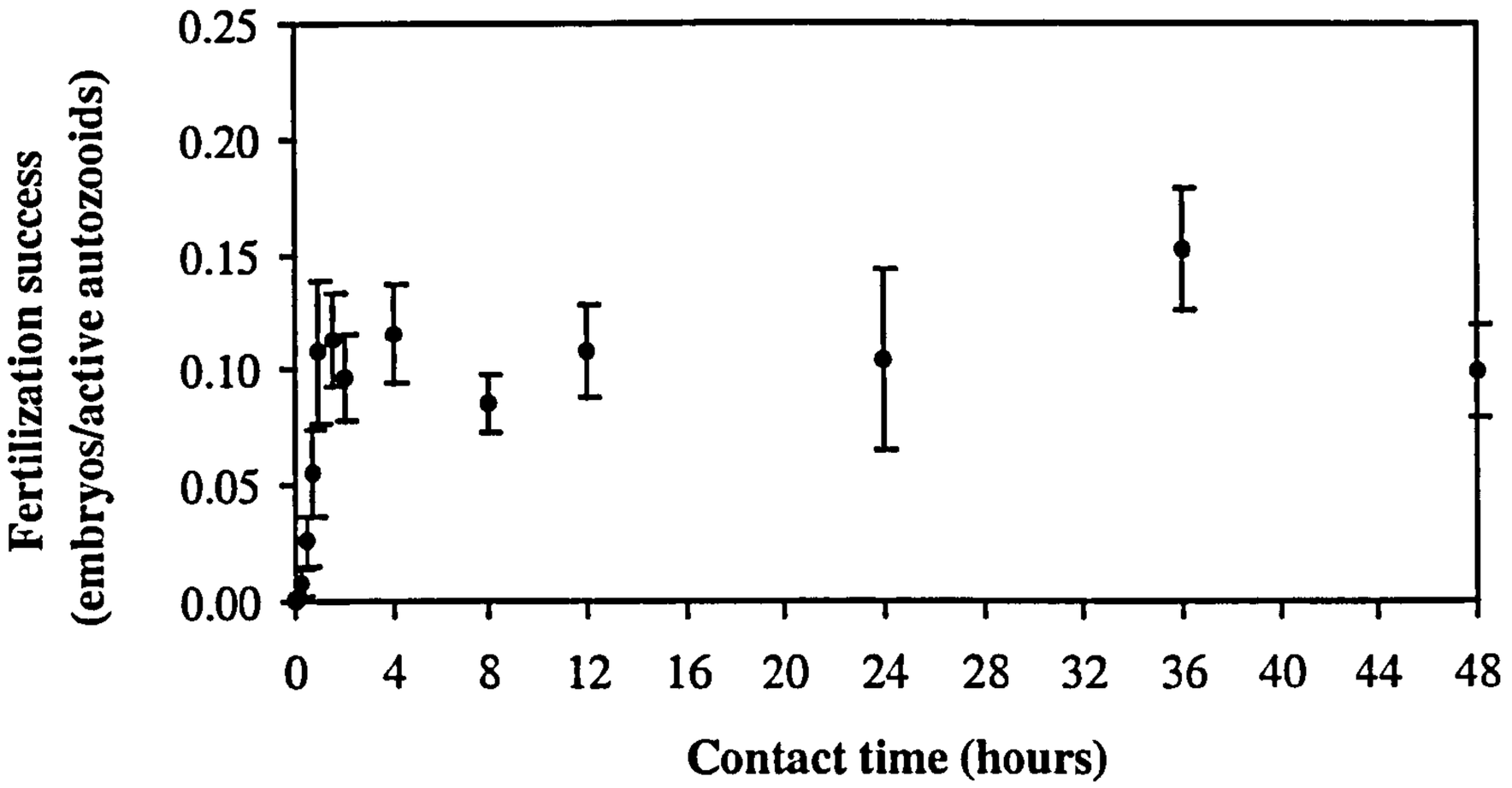


Figure 1: Fertilization success in ramets of 4 clones of C. hyalina exposed to allosperm suspensions for different contact time. Data are mean ± SD.

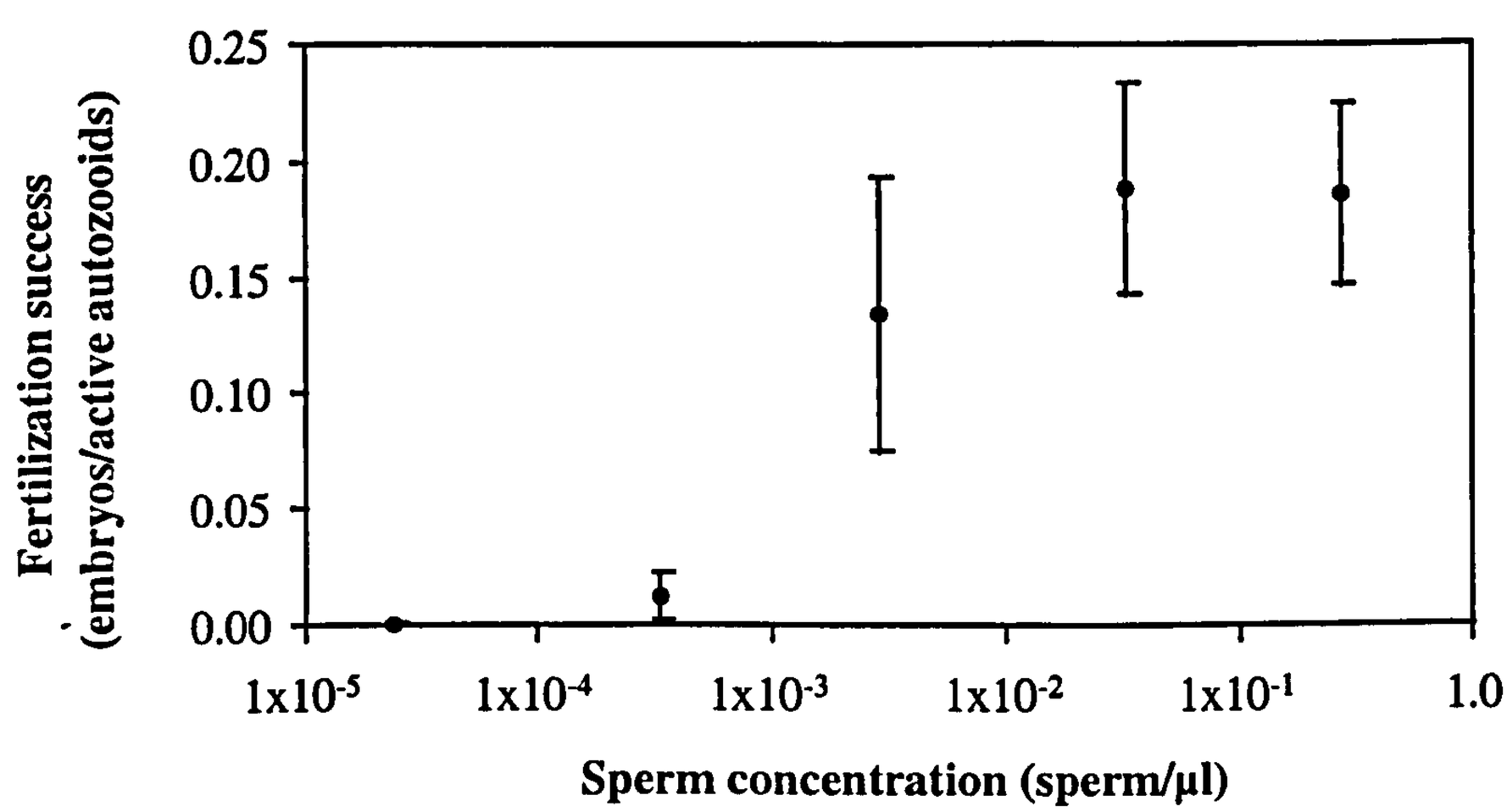


Figure 2: Fertilization success in ramets of 7 clones of C. hyalina exposed to different allosperm suspensions for 2 h. Data are mean ± SD.

After the findings of these two experiments I decided to use in all my experiments a minimum 2 h of contact between the allosperm suspensions and the receptor clones. Moreover, if sperm suspensions were one order of magnitude below $1x10^{-3}$ they were not used in the experiments, to avoid confounding effects.

Sperm counts

Aliquots of 30 ml of water from the beakers bearing the donor ramets were filtered through 25 mm diameter, 0.45 μm pore-size cellulose-nitrate membrane filters, which were placed immediately on glass microscope slides and treated with a drop of 0.1 mg ml^{-1} suspension of Hoechst 333342 stain (bis-benzimide trihydrochloride; Sigma) in distilled water. The filters were then sealed with a cover slip and nail varnish, placed under a Leitz microscope with incident UV light and sperm were counted along three vertical and three horizontal 20 mm transects across the filter, using an eyepiece graticule. Sperm counts per transects were used for estimation of total number of sperm in the filters and then to estimate number of sperm per μl. Using this protocol sperm heads were clearly visualized as green and with a conical shape.

General protocol for sperm release used in this thesis:

Once the appropriate methods of sperm release and sperm counts were determined, a standard protocol of sperm release and sperm counting was adopted to be used in all the experiments of this thesis. Ramets to be used as sources of allosperm were maintained in a 2 l beaker of aged, 0.2 μm-filtered, UV-treated, magnetically stirred FSW. The beaker stood in a water bath set at the experimental temperature. After 12 h in darkness, the ramets were transferred to a similar, but illuminated beaker. Once the colonies of allosperm donors were exposed to light, the first water samples were taken after 15 minutes. If presence of allosperm was not detected at the desired level, the water in the beaker was changed and the colonies sprayed with FSW to remove sperm. This procedure was repeated four times until allosperm were found in all the beakers bearing the ramets from the clones from which sperm were required. In the eventuality of absence of allosperm from any clone, the colonies of this clone were returned to darkness and sperm triggering attempted two days later. Once sperm where detected in all the beakers, sperm concentrations of each donor were worked out as soon as was possible in order to avoid sperm ageing. When receptor colonies were simultaneously exposed to allosperm suspensions from 2 or 3 different clones, the original suspensions were diluted using FSW to obtain similar sperm concentrations.

Appendix 3

Determination of number of basal autozooids from colony size and categorization of the experimental clones as normally or slowly growing.

When counts of the number of basal autozooids could not be made directly, measurements of colony size were employed. Camera lucida drawings have been used before to estimate number of basal zooids in C. hyalina (Cancino, 1983, McCartney, 1994). However, since I needed to visualize the time scale of my future experiment, I did my own observations in order to obtain together with the standard curve, estimations of growth under my experimental rearing conditions. Therefore throughout this thesis, camera lucida drawings were analized with an electronic digitizer and a standard curve relating number of basal zooids to colony was obtained. Weekly camera lucida drawings of 25 colonies established in April of 1996, and 25 colonies established in August of 1996 (see Appendix 1) starting from 3-4 zooid-size colonies combined with direct basal countings were used to calibrate the curve. Number and intracolonial position of each sexual zooid in the colony, i.e. frontal males, basal males and females zooids, were also included in the drawings.

During rearing, colonies were maintained in individual flat-bottomed glass vessels and the water movement provided by oscillating paddle system (Appendix 1). Food was provided daily at concentrations suggested by Hunter and Hughes (1983a, b), water change every second day and the colonies cleaned once a week.

With the purpose of identifying these colonies as a function of their characteristic growth, colonies were assigned to one of the following categories: a) normally growing genotypes and b) slowly growing genotypes. In order to evaluate if conclusions drawn from these observations were an artefact of the rearing proceedings, doubling time and reproductive allocation (i.e. number of sexual zooids to basal autozooids) were evaluated in 3 groups of 3 colonial fragments generated from each genotype during the cloning procedure (see Appendix 1). Each fragment was reduced to 10 active basal zooids and the number of basal zooids and reproductive zooids evaluated 2 wk later. Doubling time (DT) was calculated as: $DT = \ln 2/r$, where r (i.e. specific growth) was

calculated as: $r = \ln(N/N_0)/t$, where N is the colony size in zooids at a given time, and N_0 is the colony size at time = 0. Reproductive allocation was calculated, by dividing the number of sexual zooids (i.e. males and females) by the number of autozooids.

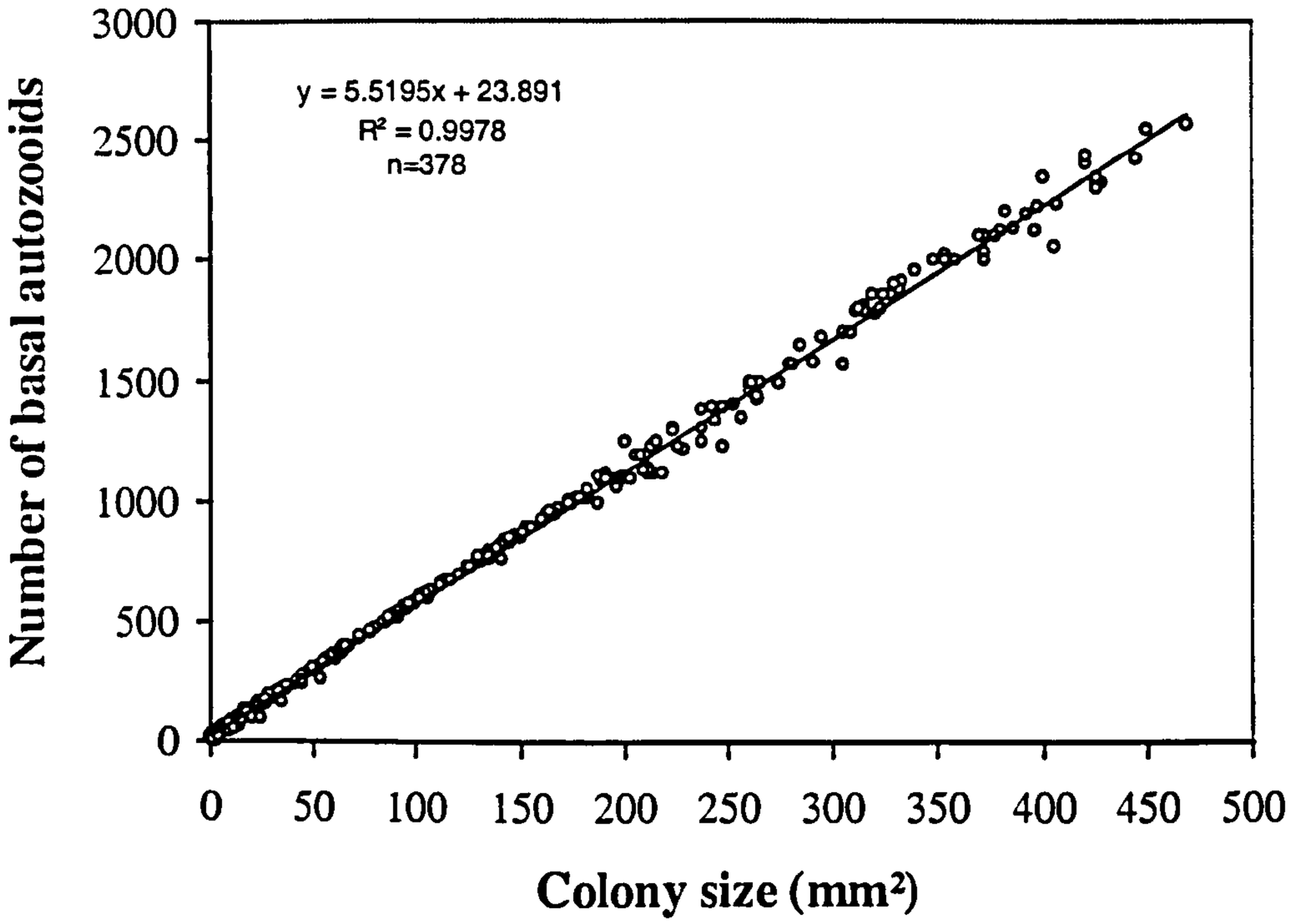


Figure 1: Relationship among colony size (are in mm^2) and number of basal autozooids in colonies of C. hyalina.

Table 1: Doubling time and reproductive allocation in 25 clones of Celleporella hyalina established in April 1996 (First set, clones A_1 to Z_1) from ancestrulae and from 10 basal autoozooids. In this set the clone T_2 was found as slowing growing clone.

From ancestrulae	Doubling time	Reproductive allocation
Normally growing clones	3.23 ± 0.12	0
Slowly growing clone	6.03	0
From 10 basal autozooids	**Doubling time**	**Reproductive allocation**
Normally growing clones		0
group 1	2.56 ± 0.10	0.04 ± 0.03
group 2	2.52 ± 0.09	0.04 ± 0.02
group 3	2.51 ± 0.06	0.04 ± 0.02
Slowly growing clone		
group 1	5.42	0.81
group 2	4.05	0.81
group 3	4.42	0.89

Table 2: Doubling time and reproductive allocation in 25 clones of Celleporella hyalina established in October 1996 (Second set, clones A_2 to Z_2) starting from ancestrulae and from 10 basal autozooids. In this set only the clone F_2 and T_2 were considered as slowing growing clones.

From ancestrulae	Doubling time	Reproductive allocation
Normally growing clones	3.21 ± 0.10	0
Slowly growing clones	7.43 ± 1.98	0
From 10 basal autozooids	**Doubling time**	**Reproductive allocation**
Normally growing clones		0
group 1	2.57 ± 0.05	0.03 ± 0.02
group 2	2.52 ± 0.06	0.04 ± 0.02
group 3	2.54 ± 0.07	0.07 ± 0.02
Slowly growing clones		
group 1	4.23 ± 0.26	1.23 ± 0.26
group 2	3.96 ± 0.65	0.94 ± 0.02
group 3	3.79 ± 0.16	1.09 ± 0.13

From Table 1 and Table 2 it seems to be clear that clones T_1, T_2 and F_2 can be assigned to the category of slow growing genotypes. Considering data from colonies of 10 basal zooids, the doubling time (ranged from 3.79 to 5.42) was approximately twice the double time recorded in the normally growing clones (ranged from 2.51 to 2.57). Both doubling times are lower than similar described before for the species (Hunter and Hughes, 1983a; Riisgård and Goldson, 1997). Moreover, highest doubling times were associated with high values of reproductive allocation. About 10-15 times more sexual zooids per basal autozooids were recorded in slowly growing clones than in the normal ones. In colonies with high values of reproductive allocation, the presence of female zooids was even recorded in anomalous basal budding, which sometimes stopped further budding in this point. In all the experiments where extensive numbers of colonies were required, normally growing clones were used. However, slowly growing clones were used for comparative experiments. When time was limiting, number of basal zooids was obtained the equation described above.

Appendix 4
Fusion and inter-colonial sperm movement

Intra-colonial fusion between two fragments of the same colony has been demonstrated in C. hyalina under laboratory conditions (see Chapter 7 and 9). Moreover, observations on colonies of C. hyalina growing on their natural substratum show that fusion between two fragments of the same colony it is possible (own observations). Similarly, fusion between the growing edges of one colony occurs when they have made contact, so restoring circularity (Cancino, 1983, own observations). The presence of sperm inside sexual zooids and feeding zooids in C. hyalina by Marcus (1938), have been interpreted as evidence of intracolonial sperm movement. These findings have been complemented by evidence of progeny being produced by female zooids budded beyond the initial colonial growing edge, i.e. the margin of receptor colonies at the moment exposure to allosperm took place. Since allosperm could be transported inside a penetrated colony (see Chapter 3), and iso-contact leads to colonial fusion (see Chapter 9), the present experiment was designed to investigate is sperm could be transported between two fused colonies.

In the present study 3 treatments were used. The Treatment 1 was designed to investigate if sperm captured by a colony are able to be stored and then transported from the penetrated colony to another colony once the colonies have made contact and fused. One receptor colony was exposed to a compatible allosperm suspension and then cleaned for 5 min with FSW. Then, using a scissors the colony was trimmed from the acetate and a small margin (ca. 1000 µm) was left surrounding the colony. The plastic bearing the colony was attached to a piece of acetate sheet (ca. 7.5 x 4.5 mm). Then, another ramet similar in size was trimmed from its acetate substratum and attached close to the colony exposed to allosperm suspensions. A small gap of 6-8 mm was kept between the two colonies. The acetates bearing the colonies were vertically dipped into the allosperm suspension (Fig. 1). Only one colony was in contact with the allosperm suspension, maintaining the level of the allosperm suspension in line with lower margin of the cut in the acetate. During the time of exposure to allosperm suspension, the top colony was exposed to desiccation. In this treatment, presence of progeny in the top colonies was considered as product of sperm moving vertically from the allosperm suspension to the top colony in the water layer associated with the acetate substratum.

In the Treatment 2. 3 couples of fused ramets with a common colliding zone of about 3 cm long were used. A slide of acetate (ca. 4 mm) was removed from the colliding zone of 3 fused colonies. Only a small piece of acetate beyond the growing edge of the colonies was maintained in order to kept the colonies onto a common substratum (Fig. 1). This treatment was used as control for sperm being moving associated to water attached to the substratum.

In treatment 3, 3 couples of fused ramets were used. As in the previous experiment only one of the colonies was vertically dipped into the allosperm suspensions. However, a physical barrier to sperm moving throughout the water tension from the allosperm suspension to the non-exposed colony was used. In the present experiment, a 3-5 mm layer of colourless liquid paraffin was poured into the container bearing the allosperm suspension (Fig.1). Once the two layered system was established, with the paraffin on top of the allosperm suspensions, the acetates bearing the fused colonies were dipped into the suspension. Care was taken to avoid the top colony touching the allosperm suspension. In preliminary tests short exposure to the liquid paraffin was shown to be non-toxic for colonies of C. hyalina.

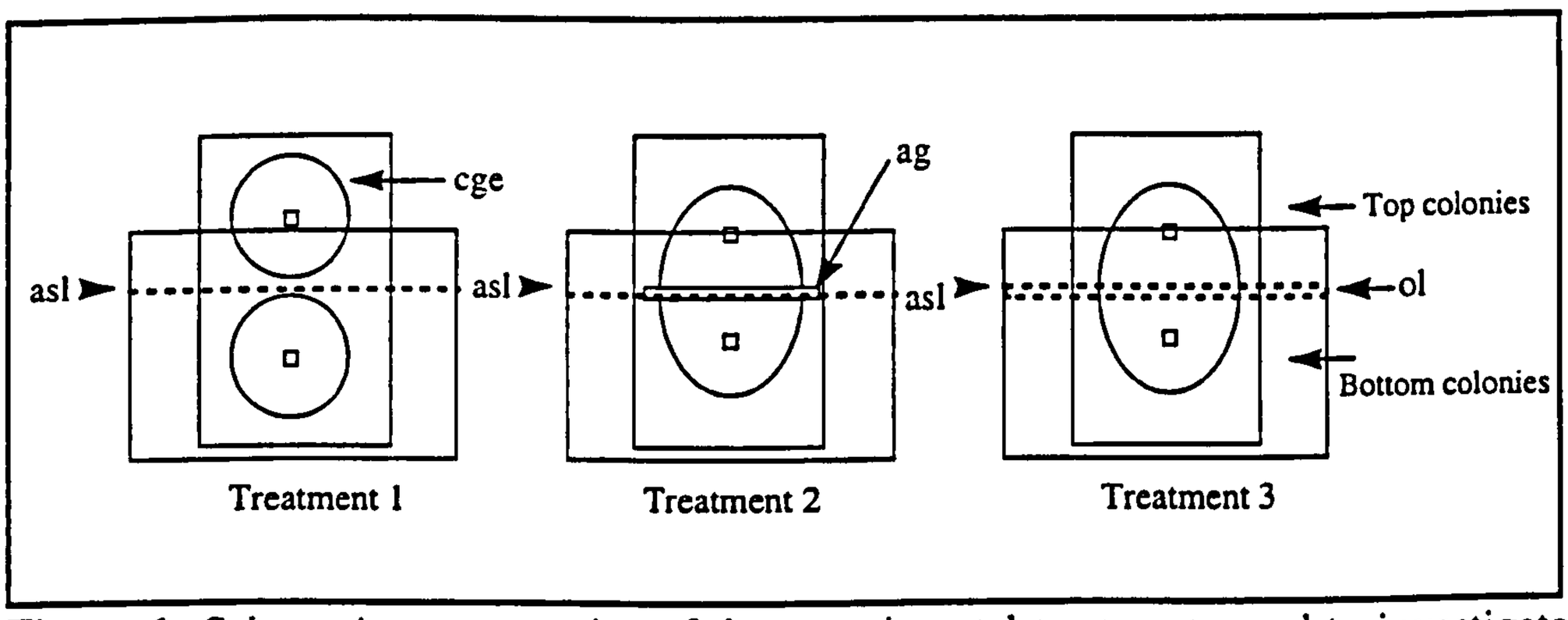


Figure 1: Schematic representation of the experimental treatments used to investigate inter-colonial allosperm movement in fused colonies of C. hyalina. cge= colonial growing edge; asl= allosperm suspension level; ag= gap in the plastic acetate; ol= liquid paraffin level on top of the allosperm suspensions. The□ inside the colonies denote the initial fragments of acetate bearing the founder zooids from which the experimental colonies were originated.

In all the treatments, colonies were maintained vertically exposed to allosperm suspensions by holding them from a plastic file holder placed across the top of the

container bearing the allosperm suspension. File holder were maintained in place by using pieces of bluetack®. Since the colonies were dipped vertically into the allosperm suspensions the other colonies on the acetate were exposed to desiccation. In order to diminish stress associated to desiccation, exposure to allosperm last only for 15 minutes. Moreover, cling film was used to cover the container bearing the allosperm suspension. After allosperm exposure, the colonies were rinsed with FSW for 5 minutes and then placed in plastic histologic racks. Colonies from each treatment were assigned to individual racks and located in separated PET bottles and maintained in standard culture conditions (see Appendix 1) and under reproductive isolation.

Once embryos were observed in the ovicells a clean, sterilize needle was used to carefully crack the ovicells. Then late embryos were removed by suction using a Gilson® pipette. To avoid trapping embryos on the walls of the pipette tip, large diameter tips were used. Collected embryos were placed into wells of a microwell plate and then placed in Eppendorf micro tubes with 10 µl of TE. For long term storage, the micro tubes bearing the samples were maintained at -80°C.

Results

Embryos were found only in the ramets directly exposed to allosperm suspensions. In treatment 1, 4 weeks after allosperm suspension no more oocytes were visible in the colonies. During this period of time embryos were only produced by the colonies directly exposed to allosperm suspensions. Oocytes and embryos were not found in the contiguous colonies not exposed to allosperm suspensions. As in treatment 1, in treatment 2 and 3 no more oocytes were visible in the colonies 4 weeks after that exposure to allosperm took place. In Treatment 2, embryos were only produced by the colonies directly exposed to allosperm suspensions. No oocytes or embryos were found in the top colonies not exposed to allosperm suspensions. In treatment 3, most embryos were found in the colonies directly exposed to allosperm suspensions. However, a few embryos (2 or 3) were also found above the top level of the liquid paraffin barrier. Usually, those embryos were 1000 µm above the level of the paraffin. The genetic fingerprint of those embryos showed that they were all fathered by the allosperm used in the exposure. No evidence of unwanted contamination of self fertilization was found in

this analysis.

Absence of progeny in colonies not exposed to allosperm suspensions showed clearly that self fertiliation is rare in C. hyalina. This result it is in agreement with those from colonies of C. hyalina maintained in reproductive isolation as control in the experiments described in the present dissertation. As progeny was not present in the colonies not exposed to allosperm suspensions growing close to or fused with an allosperm-penetrated colony, this suggests that allosperm are not leaving the penetrated colonies. This result it is in agreement with other described in this dissertation in connection with intra-colonial experiments (see Chapter 3). Despite in Treatment 1 the colonies made contact before the last oocyte were visible in the female coelom, no progeny was found in the non-exposed colonies. A possible explanation for this result is the absence of real fusion between the colliding colonies. By the time the last larvae were observed leaving the colonies no coalescent zooids were yet formed between the colliding colonies. The results of Treatment 2 confirm results of Treatment 1. Progeny was only produced by the colonies exposed to allosperm suspensions. The results of Treatment 3 showed clearly that progeny was produced by female zooids located above the physical barrier used to stop sperm movement through the surface of the colonies. There are several possible explanations for this result. It could be due to allosperm being trapped in the surface of the colonies exposed to allosperm and not being removed with the FSW rinse, unwanted contamination by allosperm entering in the PET bottles during the rearing, or by sperm being released by the penetrated colony and then re-captured by the contiguous colony. Genetic fingerprints showed that progeny was fathered by the experimental allosperm suspension, and so contamination during the rearing should be discarded. Since, progeny was not found in the colonies growing close to or in contact with an allosperm-penetrated colony, the possibility of sperm being re-captured should be also discarded. It is not possible to distinguish between progeny being fathered by allosperm trapped in the colony surface and those being moved from one colony to another through inter-colonial movement. Since progeny produced by the colonies not exposed to allosperm suspensions were located only in zooids close to the upper limit of the physical barrier to sperm movement, the results of the present experiment suggest that inter-colonial sperm movement took place. However, the possibility of fertilization by allosperm trapped in the colonial surface can not be totally discarded.

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