INTRODUCTION

Diverse marine organisms, from algae through invertebrates to fish, are free-spawners, releasing gametes into the water (Levitan 1995). Free-spawned gametes are inevitably diluted as water currents transport them away from the source; consequently they become less likely to encounter potential partners (Yund 1990). Reduced fertilisation success resulting from gametic dilution is well documented (e.g. Oliver & Babcock 1992, Coma & Lasker 1997a, Babcock et al. 2000, Yund 2000). Putatively adaptive mechanisms that counteract dilution include the close spacing of potential mates (Robertson 1996), synchronised spawning (Coma & Lasker 1997b), selection of favourable hydrographic conditions (Petersen et al. 1992, Serrão et al. 1996), limitation of gametic dispersal (Scaggiante et al. 1999), increasing target (egg) size (Levitan 1996, Styan 1998, Marshall et al. 2000) and prolonged fertility of sperm during the dispersal phase (Benzie & Dixon 1994) (for a general review see Yund 2000). Moreover, species that free-spawn sperm but retain eggs could potentially increase fertilisation success by efficiently capturing and storing water-borne sperm (Temkin 1996, Bishop 1998). Relatively prolonged fertility of water-borne sperm would further enhance fertilisation success in such organisms. This has been demonstrated in the ascidian Diplosoma listerianum (Bishop 1998) and, to assess the generality of this principle, we examined the longevity of water-borne sperm in the cheilostome bryozoan Celleporella hyalina (L.).

Bryozoans receive water-borne sperm that become entrained in the feeding current generated by the lophophore, whence they pass through the intertentacular organ/dorsal coelomopore into the coelomic cavity (Temkin 1994). Celleporella hyalina can store a batch of allosperm for several weeks and use it to fer-

Age-dependent loss of fertility in water-borne sperm of the bryozoan Celleporella hyalina

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ABSTRACT: The cheilostome bryozoan Celleporella hyalina (L.) was used as a model to test the hypothesis that sessile animals fertilising retained eggs by water-borne sperm should produce sperm with relatively prolonged fertility during the dispersal phase. Ramets of each of several recipient clones were exposed to suspensions of allosperm obtained by induced release from ramets of donor clones. Allosperm was presented to each series of ramets at a concentration of 10 to 10^2 ml^-1, in aliquots of increasing age after release. Control ramets of the recipient clones isolated from allosperm failed to produce embryos, indicating the absence of autogamy. Experimental ramets exposed to fresh allosperm copiously produced embryos, but the frequency of embryos declined sharply in ramets exposed to allosperm suspension of increasing age. The estimated fertile half life of water-borne sperm was 1.2 h, which is at the upper limit of the range of values reported for other marine animals at comparable concentration. C. hyalina maximises fertilisation success by efficient dissemination, capture and storage of relatively long-lived sperm.

KEY WORDS: Free-spawning · Allosperm · Allogamy · Sperm storage · Cloning

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utilise a series of eggs (Manríquez 1999). This paper addresses ageing of sperm in seawater, which we compare to the equivalent process in broadcast-spawning species. Subsequent ageing of stored sperm within the recipient colony does occur but is a different, and apparently much slower, process (with no obvious equivalent in broadcast spawners); this will be discussed elsewhere.

MATERIALS AND METHODS

In April 1996, colonies of Celleporella hyalina, epiphytic on Laminaria saccharina, were collected from the Menai Strait, Anglesey, North Wales (latitude 53° 16', longitude 04° 06', Ordnance Survey co-ordinates SH 609767). Fronds bearing colonies were placed in a 40 l plastic tray filled with seawater, together with acetate sheets that had been preconditioned by steeping in seawater for 1 wk. Rhinomonas reticulata was supplied as food. After 24 h in darkness, the tray was illuminated with overhead fluorescent lighting, thus inducing larval release (Ryland 1960). Settlement readily occurred on the floating acetate sheets. Newly developing colonies were isolated individually by cutting the acetate with scissors. Each colony was cloned by successively growing it to a suitable size and taking cuttings to produce a set of ramets (Manríquez 1999).

Ramets were maintained either as batches of clone-mates to serve as sperm donors or as isolated colonies to serve as sperm recipients. All ramets were strictly isolated from sources of allosperm. Batches of donors were maintained in plastic (polyethylene terephthalate), 2 l commercial drinks bottles, each modified to receive a wide plastic lid (Fig. 1a). The lid was fitted with 2 pieces of plastic delivery tubing. One tube received an aeration line fitted with a diffuser and the other received a plastic pipette whose perforated bulb, loosely plugged with cotton wool, served as an aeration vent. Ramets, chosen to exceed 20 mm in diameter, were carried in a histological staining rack placed within the bottle. Recipient ramets, chosen to be of similar size and having >60% of autozooids in the active state, were each maintained in a separate 300 ml glass jar. A plastic lid was fitted with 3 ports constructed from modified plastic micro-centrifuge tubes (Fig. 1b). One tube received a plastic pipette, whose bulb placed near to the bottom of the jar was perforated to serve as an aeration diffuser. Another tube, with a finely perforated apex and loosely plugged with cotton wool, served as an aeration vent. The third tube, retaining its lid, served as a feeding port. This system prevented any cross-contamination by sperm carried by spilled water droplets or by aerosol. Bottles and jars, two-thirds filled with 0.2 µm-filtered, UV-irradiated seawater, were housed in a room maintained at 16 ± 2°C. A sufficient volume of Rhinomonas reticulata suspension was added daily to maintain a concentration of about 10^5 cells ml⁻¹. Water was renewed every 2 d, and the colonies and substratum were cleaned with a soft artist’s brush at 1 wk intervals.

From each donor clone, sperm suspension was obtained by placing 10 ramets in a 2 l glass beaker filled with aged, 0.2 µm-filtered, UV-irradiated seawater, were housed in a room maintained at 16 ± 2°C. A sufficient volume of Rhinomonas reticulata suspension was added daily to maintain a concentration of about 10^5 cells ml⁻¹. Water was renewed every 2 d, and the colonies and substratum were cleaned with a soft artist’s brush at 1 wk intervals.

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fell below the 10 ml$^{-1}$ required for maximum fertilisation potential (Manríquez 1999), the water in the beaker was renewed and the ramets washed with fresh seawater to remove old sperm. A second sample was taken after 15 min and the process repeated up to 4 times. Ramets were returned to darkness and the procedure repeated after 2 d if satisfactory sperm release had not occurred by this stage. Aliquots of allosperm suspension used in the experiments had concentrations in the range of 10$^2$ to 10$^3$ ml$^{-1}$. Suspended allosperm had an age of 10 to 25 min (0.17 to 0.42 h) when first administered to recipients, since the period of release was 15 min and counting then took 10 min.

Mating compatibility of experimental clones A, D, E, J, M and Q was verified by immersing a ramet of each clone for 12 h in 250 ml of allosperm suspension from each of the other clones, together with 0.5 ml of *Rhinomonas reticulata* suspension as a source of food. Two controls for autogamy (self-fertilization) were run. First, a ramet from each clone was kept in isolation. Second, a ramet from each clone was immersed in autosperm suspension obtained from clonemates by standard protocol (above).

Thirteen ramets from each of clones E, F, J and Q, were placed in histological staining racks and each rack immersed in 1.5 l of stirred allosperm suspension from clone A, to ascertain the shortest exposure time required for achieving maximum fertilisation potential. About 5 ml of *Rhinomonas reticulata* suspension was added to sustain the recipient ramets. One ramet from each recipient clone was removed over a series of time intervals graded from 1 min to 48 h. The ramet was rinsed in fresh seawater for 5 min to remove residual allosperm and placed in a separate 300 ml culture jar (above). After 1 wk, ramets of each recipient clone, labelled according to exposure time, were placed in a histological staining rack and transferred to a 2 l culture bottle (above). The number of ovicells containing embryos in each ramet was counted under a dissection microscope at intervals of 3 d over a period of 2 mo. Fertilisation success was expressed as the cumulative number of embryos per gynozooid. It should be noted that new gynozooids were produced as the recipient ramets grew during the observation period and were fertilised by stored allosperm. Moreover, gynozooids present initially could produce 2 to 3 successive embryos. A control ramet from each recipient clone was kept free from allosperm.

Two experiments estimating the half-life of suspended sperm were run. The first experiment investigated temporal loss of fertility of sperm at 16°C. The following clones were paired: donor/recipient = M/A, A/E, J/Q and Q/D. Seventeen ramets of each recipient clone were exposed to allosperm suspension of known age, ranging from 0.17 to 48 h. To do this, 250 ml of allosperm suspension were removed from the source vessel (above). An aliquot of 30 ml was used for sperm counting and the remaining 220 ml were placed in a 250 ml glass beaker. The recipient ramet was immersed in the allosperm suspension for 1 h and then rinsed in fresh seawater for 5 min before being transferred to its culture vessel (above). Production of embryos was monitored for 2 mo (above). Fertility was measured as above. The second experiment investigated the effect of temperature on loss of fertility. Donor/recipient clones were M/A and A/E. Fifteen ramets of each recipient clone were randomly assigned to 3 beakers standing in water baths at temperatures of 12, 16 and 18°C. Other procedures were as in Expt 1. In both experiments, a ramet from each recipient clone was kept isolated from allosperm sources, to control for autogamy.

For statistical analysis, trailing zeros were excluded and data were standardised as proportions of the initial value for each recipient clone. Analysis of covariance (ANOVA) was applied, using ln(fertility) as the response variable and ln(age of allosperm suspension) as the covariate.

**RESULTS**

No control ramet in any trial developed embryos, confirming the absence of autogamy (self-fertilisation). All embryos resulting from exposure to allosperm therefore could be regarded as the product of allogamy (outcrossing). In the compatibility trials, recipients of allosperm readily developed embryos, confirming mating compatibility between all pairs. In the trials on exposure time, fertilisation success became asymptotic after immersion for about 1 h in allosperm suspension (Fig. 2), this being subsequently adopted as the standard exposure time.

Sperm half-life Expt 1: None of the control ramets developed embryos, thus confirming the absence of autogamy. Ramets exposed to fresh allosperm suspen-
sion copiously produced embryos, but output declined sharply in ramets exposed to allosperm of increasing age (Fig. 3). With one exception, ramets exposed to allosperm older than 12 h failed to produce embryos. A ramet of clone D, exposed to allosperm approximately 24 h in age, produced 1 embryo. The rate of decline in fertility with age of the allosperm suspension was not significantly different among clones (ANOVA, clone \( \times \ln(\text{time}) F_{3,31} = 1.34, p = 0.279, \text{power} = 0.321 \)). Parameters for the common regression of ln(fertility) on ln(age of allosperm suspension) were: intercept = \(-0.4882 \pm 0.0782\), slope = \(-1.0572 \pm 0.0662\). Using this equation for back calculation, the fertile half-life of suspended allosperm was estimated to be 1.21 h.

Sperm half-life Expt 2: Temperature did not significantly influence the rate of decline in fertility of suspended allosperm (ANOVA, temperature \( \times \ln(\text{time}) F_{2,34} = 1.69, p = 0.201, \text{power} = 0.330 \)). Back calculation from the common regression equation estimated the half-life of the allosperm suspension to be 1.24 h.

**DISCUSSION**

The progressive accumulation of fertilisations over 1 h of sperm exposure in *Celleporella hyalina* is in contrast to the results of several laboratory studies of external fertilisation, in which most fertilisations occurred within the first minute (Rothschild & Swann 1951, Levitan et al. 1991, Babock et al. 1994, Trippel & Morgan 1994, Babcock & Keesing 1999). On the other hand, Benzie & Dixon (1994) and Lasker et al. (1996) reported that considerably more than 1 min of exposure to sperm could be required to ensure high external fertilisation rates in their experiments. The pattern in *C. hyalina* presumably reflects the gradual accumulation of sperm from dilute suspension by the recipient bryozoan and is made possible, under the conditions of the experiment where progressive dilution is prevented, by the relatively long half-life of sperm.

*Celleporella hyalina* sperm are motile when newly released into fresh seawater (P.H.M. pers. obs.) and so there appears to be no energy-saving device promoting longevity, such as the suppression of motility until activation by egg exudates recorded in the ascidians *Ciona intestinalis* and *Ascidiella aspersa* (Bolton & Havenhand 1996). This interpretation must be treated with caution, however, since Temkin (1991) described quiescence of sperm of the bryozoan *Membranipora membranacea* after initial swimming, with reactivation upon entry into a lophophore.

Our failure to detect an effect of temperature on sperm longevity, although perhaps reflecting an insufficient differential between treatments, nevertheless matches the absence of any clear trend among published data. Benzie & Dixon’s (1994) experiments on *Acanthaster planci*, which indicated very long-lived sperm, where done at the highest temperature (28°C) of the studies included in Fig. 4. *Haliotis tuberculata* (Baker & Tyler 2001) was studied at 19°C and had short-lived sperm. The studies on *Strongylocentrotus* spp. by Levitan (1993) were done at 9°C and indicated intermediate longevities. It thus seems improbable that
a coherent overall trend with temperature would emerge from a cross-species analysis of the available data.

The observed half-life of about 1 h for water-borne sperm of *Celleporella hyalina* is considerably shorter than the corresponding value of 8 h for the ascidian *Diplosoma listerianum* (Bishop 1998), yet lies near the maximum recorded at comparable sperm concentrations for other free-spawners (Fig. 4). *C. hyalina*, like *D. listerianum*, therefore appears to promote fertilisation potential through the longevity of water-borne sperm.

The sperm concentration used in the present study lies at the lower edge of the published range (Fig. 4) where, as a result of the respiratory-dilution effect, sperm longevity is expected to be shorter than at higher concentrations (Chia & Bickell 1983, Benzie & Dixon 1994, Babcock & Keessing 1999). Rapid attenuation of fertilisation success with increasing distance between adults is now an established paradigm for free-spawners (e.g. Pennington 1985, Yund 1990, 2000 Grosberg 1991, Brazeau & Lasker 1992, Benzie et al. 1994). No information is available on *Celleporella hyalina* sperm concentrations in the field, but sperm suspensions must become rapidly diluted after release. On the other hand, synchronous release of sperm clouds by the protruded male lophophores within a colony has been recorded under both stagnant and low-flow conditions (Manriquez 1999). In the present study, male lophophores were observed bending toward and releasing sperm into the exhalant currents of adjacent feeding lophophores, possibly promoting dispersal in a similar way to that proposed for *Membranipora membranacea*, but which utilises the combined exhalant current of groups of zooids (Temkin 1994). Intra-colonial synchrony in release of sperm during periods of low water flow may help to compensate for dilution and limited duration of sperm fertility. Moreover, the propensity of *C. hyalina* to colonize closely spaced algal fronds may limit the effect of sperm dilution through local reductions in water flow, as demonstrated in the sea urchin *Strongylocentrotus droebachiensis* spawning beneath algal canopy (Wahle & Peckham 1999).

Lack of embryo production by isolated control colonies in the present study confirms other work (Hoare et al. 1999) showing that in Welsh populations of *Celleporella hyalina*, most colonies are virtually incapable of successful autogamy. In this respect they seemingly differ from eastern North American colonies (Yund & McCartney 1994). However, under conditions of strict reproductive isolation in the Bangor laboratory, progeny from the American study population, also, do not produce embryos (Hughes et al. in press). Thus Welsh colonies, at least, appear to rely predominately on efficient sperm dissemination, capture and storage to maximise fertilisation success and this may well be true of *C. hyalina* in general.

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**LITERATURE CITED**


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