The Royal Society

Fertilization in the sea: are the hazards of broadcast spawning avoided when free-spawned sperm fertilize retained eggs?

J. D. D. Bishop

Marine Biological Association of the UK, Citadel Hill, Plymouth, Devon PL1 2PB, UK (jbis@mba.ac.uk)

In broadcast-spawning marine animals, rapid dilution and short lifespan of sperm following release may impose severely localized patterns of mating. Partial or total failure of external fertilization due to sperm limitation appears commonplace. However, it is not clear to what extent the restrictive kinetics of fertilization in water also constrain mating in animals that release sperm but retain their eggs for fertilization.

The compound ascidian *Diplosoma listerianum* liberates sperm that are dispersed to other colonies and taken in prior to internal cross-fertilization. The fertile lifespan of sperm was found to be long (half-life *ca.* 8 hours), and a substantial number of fertilizations occurred with 24-hour-old sperm. Fertilizations were obtained from sperm concentrations that would typically produce little or no external fertilization. In a separate experiment, a very small piece of *D. listerianum* (dry weight less than 2 mg) sired abundant progeny throughout a 3840 l tank. Paternity of progeny in these experiments was confirmed by molecular markers. The same markers were used to extend, to over seven weeks, the known maximum period of storage of exogenous sperm prior to fertilization in this species.

The production of only a few thousand sperm at a time by each zooid, poor synchronization of release between zooids, and the existence of many well-spaced exhalant openings in large colonies suggest that *D. listerianum* is incapable of generating a dense plume of sperm, even close to the source. It is suggested that, unlike external fertilization, successful internal cross-fertilization in *D. listerianum* is not dependent upon the interception of a dense cloud of gametes just released by a near neighbour. It seems instead that dilute, long-lived sperm can be extracted efficiently from seawater by this suspension feeder, potentially over a period of time. This capability, and other features of the life history, make it unlikely that sperm limitation is an acute problem in this species and comparable taxa, a conclusion with potential significance for expected patterns of mating, sex allocation and gamete attributes in sessile aquatic invertebrates. Variance in reproductive success between individuals due to differences in fertilization rate may be much lower than in broadcast spawners exhibiting external fertilization.

Keywords: spawning; fertilization; sperm; ascidian; *Diplosoma listerianum*

1. INTRODUCTION

Restricted longevity of sperm after release, and rapid dilution in turbulent flow, may severely limit mating distances in marine invertebrates that release their gametes, promoting behaviour such as aggregated or synchronized spawning (Pennington 1985; Denny 1988; Denny & Shibata 1989; Yund 1990; Havenhand 1991; Levitan 1991, 1995; Brazeau & Lasker 1992; Levitan *et al.* 1992; Petersen *et al.* 1992; André & Lindegarth 1995; Levitan & Young 1995; Lasker *et al.* 1996; Coma & Lasker 1997). Unless water flows are unusually weak, sperm dilution imposes more severe constraints on fertilization success than sperm longevity (Pennington 1985; Denny & Shibata 1989; Levitan *et al.* 1991; Benzie & Dixon 1994; André & Lindegarth 1995; Levitan & Young 1995). Rates of fertilization commonly vary between 0 and 100% within single species that release both sets of gametes for external fertilization (Levitan 1995; Levitan & Petersen 1995). Fertilization success depends on the proximity of potential mates and on whether dispersing clouds of eggs and sperm happen to intersect during a critical interval, possibly lasting only a matter of seconds (Denny & Shibata 1989), immediately following spawning. Sperm limitation may have important consequences for patterns of reproductive behaviour and investment, sexual dimorphism, gamete attributes, and development (Levitan 1993, 1996*a*,*b*; Craig *et al.* 1997).

Although external fertilization of spawned eggs is common in the sea, it is by no means universal. A wide variety of sessile marine animals retain their eggs and release only sperm into the surrounding water. Sperm are then taken up for fertilization, and embryos are often brooded. Amongst sessile modular invertebrates, retention of eggs is seen in many sponges and cnidarians, and in almost all bryozoans and colonial ascidians. This reproductive pattern is also found in certain unitary sessile forms, including some bivalve molluscs and tubicolous polychaetes. Spärck (1927) proposed that fertilization would present particular problems for species that release

Received 21 November 1997 *Accepted* 16 December 1997

sperm but restrict access to their eggs by retaining them for fertilization. However, recently it has been suggested that the brooding of eggs might facilitate cross-fertilization when combined with active accumulation, and in some cases storage, of sperm from dilute suspensions (Denny & Shibata 1989; Bishop & Ryland 1991; Ryland & Bishop 1993; Miller 1994; Levitan & Petersen 1995; Yund 1995; Temkin 1996; McCartney 1997). In either case, movement of sperm prior to fertilization may contribute significantly to the limited gene flow experienced by sessile species that brood embryos and release short-lived larvae (Grosberg 1991; Yund 1990, 1995).

The didemnid ascidian *Diplosoma listerianum* (Milne Edwards) grows by asexual budding into a colony of small (*ca.* 1.5 mm) zooids. Sperm released by the individual zooids leave the colony in the exhalant current and rapidly disperse into the surrounding water (Bishop & Ryland 1991). Entering another colony, sperm move up the fertilization canal (oviduct) of a zooid into the lumen of the ovary (Burighel *et al.* 1986; Burighel & Martinucci 1994*a*; Bishop & Sommerfeldt 1996), where they may be stored (Bishop & Ryland 1991; Burighel & Martinucci 1994*a*; Bishop & Sommerfeldt 1996). True internal fertilization occurs around the time of ovulation of the fully-grown oocyte directly into the colonial tunic (Burighel & Martinucci 1994*b*), where brooding occurs. *D. listerianum* zooids are hermaphroditic, but selfing is prevented by the blocking and phagocytic destruction of self sperm in the oviduct (Bishop 1996; Bishop *et al.* 1996). Developing oocytes and brooded embryos can be observed directly in live specimens cultured on glass.

This study aims to elucidate further the process of mating in *D. listerianum*, by investigating the longevity of sperm after release and the ability of the species to obtain outcrossed fertilizations from dilute sperm suspensions. Molecular paternity markers were used to confirm outcrossing in these investigations, and to estimate the maximum duration of storage of exogenous sperm.

2. MATERIALS AND METHODS

Laboratory clones of *D. listerianum* each originated from a single larva released by a different colony from Queen's Dock, Swansea, Wales, as described by Bishop *et al.* (1996) for these and other clones. The clones were cultured in reproductive isolation, and divided into independent pieces (ramets) for experimental purposes, as described by Ryland & Bishop (1990). Crosses were achieved by placing ramets of two compatible clones in the same tank for a period, or by exposing ramets to water containing compatible released sperm.

(a) *Experiment 1A: sperm longevity*

To assess sperm longevity, fertilization was assayed by the number of late-stage embryos produced following exposure to sperm of differing ages. Clone *A* was used in the female role to receive sperm from clone *B*.

Twenty-two virgin ramets of clone *A* with 55 ± 3 zooids were each allowed to attach to a different glass microscope slide. Clone *A* does not routinely self in reproductive isolation, although small numbers of irregular early embryos are occasionally produced and aborted from the tunic (cf. Bishop & Ryland 1991). Numerous ramets of clone *B* were maintained at 16.5 °C in seven small, stirred tanks (*ca.* 830 ml volume). The water in these tanks was changed for aged, UV-sterilized natural seawater and left for 1 h. It was then collected into a single large polyethylene beaker and stirred gently on a magnetic stirrer, with suitable insulation to avoid heating of the water by the stirrer. Two 50 ml samples of this water were removed at predetermined intervals and each placed in a small plastic dish with a ramet of clone *A* for 1 h, without stirring. Each ramet of clone *A* was then given two 5-min rinses in 50 ml of UV-sterile seawater, in new containers, and transferred to stirred holding tanks in standard culture conditions. The times of sampling, starting at the completion of collection of water from the clone *B* tanks, were 0 min, 16 min, 35 min, 1 h, 2 h, 4 h, 8 h, 15 h, 24 h, 36 h, and 48 h. Ramets of clone *A* were allocated to times at random. Once all ramets had been exposed they were randomly reallocated to five standard culture tanks, each tank therefore containing four or five ramets, and maintained until scoring took place. The number of tailed (i.e. late-stage) embryos brooded within the tunic was noted 24 d and again 35 d after exposure. Larvae remain in the tunic for *ca.* 6 d after the appearance of a segmented tail (Ryland & Bishop 1990), so the two counts of tailed larvae were completely non-overlapping, and were summed.

(b) *Experiment 1B: sperm longevity*

Experiment 1A was repeated using different virgin ramets of the same clones. The experimental protocol was identical to that of experiment 1A, but an additional exposure of clone *A* ramets to water from clone *B* tanks was made 72 h after the collection of water. In addition, an attempt was made to count the sperm in suspension. At each sampling time, two 35 ml samples of water from the beaker were collected in a disposable syringe and filtered through a 25 mm diameter, 0.45 µm pore-size cellulose nitrate membrane filter (Whatman, Maidstone, UK). Two extra water samples were taken and filtered at the end of this part of the experiment (72 h) after the water remaining in the beaker had been stirred vigorously. Filters were frozen immediately for subsequent counting of sperm (see below).

Once all 24 ramets of clone *A* had been exposed, they were allocated at random to eight culture tanks, three ramets to a tank. The number of tailed embryos brooded within the tunic was noted 24 d, and again 32 d, after exposure.

(i) *Sperm counting*

Each filter was placed on a glass microscope slide, treated with one drop of 0.1 mg ml^{-1} Hoechst 33342 stain (bis-benzimide trihydrochloride; Sigma, Gillingham, Dorset, UK) in distilled water, and covered with a coverslip. The preparation was sealed around the edges with nail varnish and illuminated with incident 350–460 nm wavelength UV light from a Leitz Ploemopak illuminator (filter block G) on a Leitz Dialux 20 EB microscope in a darkened room. The distinctive needle-shaped sperm heads (*ca.* 1×14 µm; described by Tuzet *et al.* 1972; Burighel *et al.* 1985) fluoresced bright green, and the narrower tails were also discernible. Sperm were counted at $\times 400$ magnification in four complete transects passing close to the centre of each filter, two vertical and two horizontal, the width of the transect (0.273 mm) being delimited by an eyepiece graticule.

(c) *Experiment 2: sperm uptake in a large tank*

A 3.66×1.22 m tank was filled with natural seawater to a depth of 0.86 m (volume of water $= 3.84 \times 10^3$ l), with no through-flow but with gentle aeration in the middle of the tank at two points. The tank was housed in a building without temperature control. The water was left for 4 d to allow naturally

occurring *D. listerianum* sperm to die. Several litres of two different microalgal cultures were added to provide food. Twenty microscope slides, each bearing a virgin ramet of clone *F* with 60±5 zooids were allocated at random to four arrays of five slides suspended vertically in mid-water at horizontal distances of 0 mm, 228 mm, 913 mm, and 3.65 m from one end of the tank. A single ramet of clone *E*, also on a glass microscope slide, was included in the 0 mm array as the third slide in from one end. This clone *E* ramet had 101 zooids, of which 58 had discernible testes, the remaining zooids being immature. The ramets were left for 3 d, during which time the water temperature rose from 18.5 to 19.5 °C. The clone *F* ramets were then removed and allocated in random pairs to ten standard (830 ml), stirred culture tanks, subjected to two rapid water changes, and maintained at 16.5 °C. The number of tailed embryos brooded by each clone *F* ramet was recorded 15, 22 and 29 d after removal from the large tank, and the separate counts were summed.

For comparison, similar manipulations were carried out in a much smaller volume of water in the standard, stirred, culture tanks. Ten new virgin ramets of clone *F*, each with 60±5 zooids, were placed in each of two tanks containing a ramet of clone *E* with 29 mature zooids (i.e. possessing productive testes) and 768 ml of seawater. After 3 d at a temperature of 16.5 °C, ten clone *F* ramets, five from each original tank, were allocated in random pairs to five new culture tanks. They were subjected to two rapid water changes, and maintained at 16.5 °C. The number of tailed embryos was counted at the same intervals after separation from the sperm source as in the large tank trial. (The remaining ten clone *F* ramets were discarded because of restricted culture space.) Note that the ratio of clone *E* testes to clone *F* zooids during the 3 d mating period was the same as in the large tank, but the total volume of water was 2500 times smaller.

(d) *Experiment 3: observations on sperm production*

Glass Pasteur pipettes were coated with Sigmacote, followed by a dilute solution of 10 k molecular weight polyethylene glycol (both Sigma). Occasional, discrete emissions of sperm from individual zooids (cf. Bishop & Ryland 1991) were drawn into a pipette as they left the colony via the exhalant opening, and each was dispersed into 10 ml of filtered seawater in a disposable syringe. Sperm were filtered onto a 13 mm diameter, black gridded, 0.45 µm pore-size mixed cellulose ester filter (Millipore, Watford, UK), stained with one drop of 100 µg ml^{-1} DAPI (4′,6-diamidino-2-phenylindole; Sigma), sealed under a coverslip and counted under the fluorescent microscope as described for experiment 1B above.

(e) *Experiment 4: maximum duration of sperm storage*

To investigate the maximum duration of sperm storage, ramets of clones *B* and *C* were placed in the same tank and allowed to cross for 7 d, and progeny released by the clone *C* ramets were collected daily after the termination of crossing (i.e. after return to separate tanks). The last five progeny to be produced were subjected to genetic analysis using RAPD markers to confirm clone *B* paternity; these five were released within a week of each other.

(f) *Paternity analysis to confirm outcrossing*

For paternity analysis, progeny were taken as larvae or as newly-metamorphosed (two-zooid) colonies and frozen in 8 µl of 0.1 M EDTA, pH 7.9. DNA was extracted, and paternity inferred by genetic analysis using RAPD (random amplified polymorphic DNA) markers as reported by Bishop *et al.* (1996).

Progeny were not collected from experiments 1A and 1B for the genetic confirmation of outcrossing from aged sperm. Subsequently, once molecular paternity markers had been developed, ramets of a different clone, clone *C*, were exposed to water 24 h after collection from clone *B* tanks. Numerous progeny of clone *C* were produced, and 34 of these were collected and analysed for the presence of a 705 bp fragment in amplification products from decamer primer OPF-10 (Operon Technologies, Inc., Alameda, CA, USA). This marker band was not possessed by clone *C*, but was transmitted to 100% of clone *B* progeny in controlled trial matings, i.e. behaved in this context as a homozygous marker for clone *B* paternity. For experiment 4, five progeny were analysed using the same marker (OPF-10, 705 bp).

For experiment 2, 18 progeny from two ramets of clone *F* held for 3 d at a distance of 3.65 m from the source of sperm were analysed. The marker used was a 500 bp fragment from primer OPR-13, homozygous in clone *E* but absent in clone *F*.

3. RESULTS

(a) *Experiment 1: sperm longevity*

Production of tailed embryos declined following exposure to sperm of increasing age (figure 1). Approximately 2.8 times more progeny in total were produced in experiment 1A than in experiment 1B. Nevertheless, the decline in production followed a similar course in the two experiments, and was approximately linear when plotted against a logarithmic time-scale (cf. Havenhand 1991). Linear, least-squares regressions against log-transformed sperm age for experiments 1A and 1B had r^2 values of 0.67 and 0.78, and estimated sperm half-lives (times at which 50% of the initial number of embryos would be produced) of 8.2 and 7.4 hours, respectively. Several fertilizations took place with 24-hour-old sperm, and one fertilization, in experiment 1B, with 48-hour-old sperm.

Sperm counts from filtered samples of water in experiment 1B are shown in figure 2. Despite considerable noise in the data, a general progressive decline in numbers of sperm in suspension is evident. The initial sperm count was equivalent to a sperm concentration at the start of ageing of 9.3 (±1.8 s.e.) sperm ml^{-1}. Vigorous stirring of the beaker at the end of the experiment resuspended

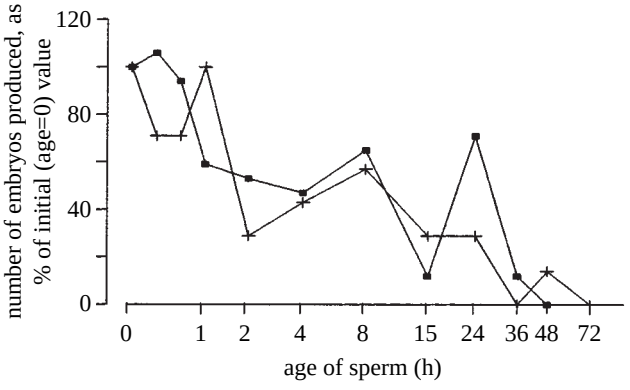

Figure 1. Production of embryos by *Diplosoma listerianum* exposed to sperm of various ages (minimum time since release; note logarithmic time-scale). Squares, experiment 1A (105 progeny in total); crosses, experiment 1B (38 progeny).

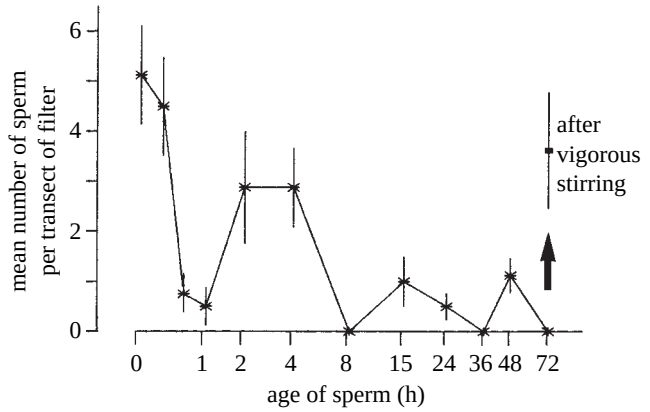

Figure 2. Number of *Diplosoma listerianum* sperm in suspension in stock beaker as function of sperm age (minimum time since release) during experiment 1B (mean counts ± 1 s.e.).

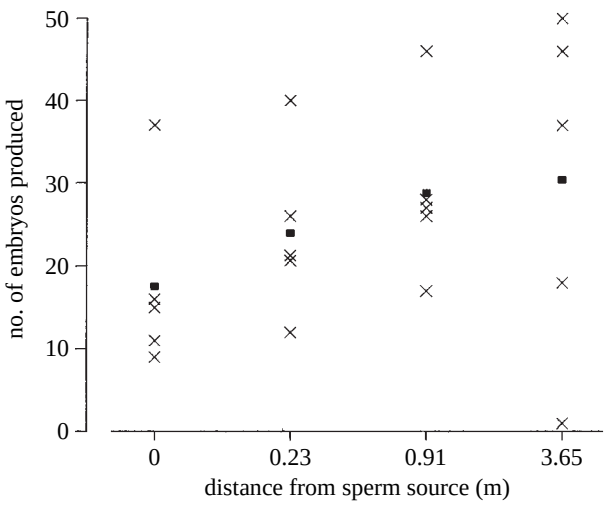

Figure 3. Number of *Diplosoma listerianum* embryos produced at various distances from 58 mature zooid sperm source during experiment 2. Squares, means; crosses, values for individual replicates (ramets).

debris that was then retained on the filter, and the sperm count was restored to a relatively high value equivalent to 6.6 (±2.1 s.e.) sperm ml^{-1}, although all sperm in these last samples were considered to be dead, showing signs of damage or deterioration, and in many cases having adherent bacteria.

RAPD analysis (cf. Bishop *et al.* 1996) revealed the 705 bp marker band in amplified DNA of all 34 progeny resulting from the exposure of clone *C* ramets to 24-hour-old clone *B* sperm, confirming the occurrence of outcrossed fertilization from aged sperm.

(b) *Experiment 2: sperm uptake in a large tank*

Numerous fertilizations occurred at all distances from the sperm source in the 3840 l tank (figure 3), with no significant overall effect of distance (Kruskal–Wallis test, $p = 0.271$ after correction for ties). Surprisingly, the mean number of embryos per ramet increased monotonically with distance from the sperm source, and this trend was almost significant (Kendall's test for ranked categories, $p = 0.086$ in two-tailed test). Three of the six most productive ramets were at the far end of the tank.

The RAPD marker band for clone *E* paternity was present in all 18 progeny analysed from clone *F* ramets at the far end of the large tank.

The clone *F* ramets in the small tank trials produced almost exactly twice as many larvae as did those in the large tank (mean number of embryos per ramet: large tank, 25.2 ± 3.1 s.e.; small tank, 51.8 ± 3.3 s.e.; *t*-test for difference between means, $p < 0.0005$).

(c) *Experiment 3: sperm production*

Estimated numbers of sperm in discrete emissions from single zooids ranged from 834 to 3415 (mean 2124 ± 262 s.e., $n = 10$). DAPI was found to be somewhat preferable as a stain to Hoechst 33342, since fading due to photobleaching was less pronounced.

(d) *Experiment 4: maximum duration of sperm storage*

Progeny were released by the clone *C* ramets from the 25th to the 68th day after separation from clone *B* following a seven day mating period. The 705 bp RAPD marker band was present in each of the last five progeny produced, confirming outcrossing from stored clone *B* sperm. Fertilization in *D. listerianum* occurs around the time of ovulation (Burighel & Martinucci 1994*b*). Given a period of embryonic incubation, between ovulation and larval release, of 13 days at the culture temperature (Ryland & Bishop 1990), the last larva resulted from a period of sperm storage prior to fertilization of *ca.* 55 days.

4. DISCUSSION

Brief fertile lifespans of several seconds to a few hours have been reported for diluted sperm of various marine animal taxa (Lillie 1915; Vogel *et al.* 1982; Pennington 1985; Grosberg 1987; Yund 1990; Petersen *et al.* 1992; Levitan 1993; Suquet *et al.* 1994; Trippel & Morgan 1994; André & Lindegarth 1995; Lasker *et al.* 1996). The lifespan of *Diplosoma listerianum* sperm reported here is considerably longer (half-life *ca.* 8 hours), and a substantial number of fertilizations still occurred with 24-hour-old sperm, while one larva was produced after 48 hours. Spermatozoa contain finite energy stores, and a trade-off may exist between longevity and level of activity, as suggested by Levitan (1993) for three species of echinoid. It is possible that energy resources of *D. listerianum* sperm are conserved by quiescence or intermittent swimming during its relatively protracted fertile lifespan. Sperm of broadcast-spawning solitary ascidians may remain quiescent upon dilution in pure seawater, being activated by substances emanating from eggs (Minganti 1951; Miller 1982; Yoshida *et al.* 1993; Morisawa 1994). Bolton & Havenhand (1996) report that sperm longevity is considerably reduced in activated sperm. In the absence of egg substances, extended periods of survival of a substantial proportion of sperm, comparable to that reported here for *D. listerianum*, have been documented in two solitary ascidian species (Harumi *et al.* 1990; Havenhand 1991). This longevity is in contrast to the brief (less than 5 minute) fertile lifespan of sperm reported in the compound ascidian *Botryllus schlosseri* (Grosberg 1987). Intermittent swimming of released sperm of internally fertilizing pelagic tunicates

(salps) has been reported, and postulated to increase sperm longevity; after uptake, sperm swim continuously in aggregations around the female gonopore (Miller 1994, 1996). Similarly, spermatozeugmata of the bryozoan *Membranipora membranacea* become quiescent after an initial swimming phase following release, but are re-activated when drawn into a lophophore (Temkin 1991).

The decline in sperm counts from water filtered during experiment 1B (figure 2) could have resulted exclusively from the natural death and sinking of sperm: a substantial number of sperm, all of which were considered to be dead, were resuspended by vigorous stirring after 72 hours. However, the decline could also have reflected the removal of live sperm from suspension by adhesion to the walls and bottom of the vessel or by entanglement in debris near the bottom. If attachment to solid surfaces did remove some live sperm from the water, the decline in fertilizations recorded would have been partly artefactual, and sperm longevity will have been underestimated. There is considerable unexplained variation in the counts of sperm during the period of ageing in experiment 1B. This variation has been less apparent in subsequent use of the method, particularly when mean counts are higher. A similar filtration technique for enumerating dilute sperm has been used by Coma & Lasker (1997).

In experiment 2 (figure 3), fertilizations did not decrease with increasing distance from the single small source of sperm in the 3840 l tank. Emissions of sperm by individual zooids of *D. listerianum* disperse almost instantly into individual gametes as they leave the colony, and release of sperm is at best poorly synchronized between zooids (Bishop & Ryland 1991; J. D. D. Bishop, present observations). In all but the smallest colonies, sperm will exit through several or many well-spaced exhalant openings rather than from a single point. Coupled with the production of only a few thousand sperm at a time by each zooid (experiment 3), these observations suggest that colonies of *D. listerianum* are incapable of generating a dense cloud of sperm, even close to the source. It is thus apparent from the results of experiments 2 and 3 that fertilization is not dependent upon the interception of a concentrated plume by near neighbours of the source colony. Abundant fertilizations can result from the uptake of sperm after it has undergone considerable transport and dilution. As has been suggested before (Denny & Shibata 1989; Ryland & Bishop 1993; Miller 1994; Temkin 1996), the particle capture systems of suspension-feeding sessile invertebrates may efficiently gather sperm, as well as food. The route of entry of sperm to the site of fertilization in larviparous ascidians appears problematical (Bishop & Ryland 1993; Ryland & Bishop 1993) and remains to be elucidated. However, Miller (1994, 1996) reports passage of sperm through the mucous-feeding mesh in pelagic tunicates, allowing access to the atrial cavity where the female gonopore is situated. It seems highly probable that fertilizing sperm also enter *D. listerianum* colonies with the inhalant current, and may thus be actively gathered.

An inferred 2500-fold decrease in sperm dilution in the small tank trial compared with the large tank trial resulted in only a two-fold increase in the number of progeny produced. These ratios suggest that the curve of embryo production versus sperm concentration was levelling off such that clone *F* ramets in the small tanks were at, or very close to, their maximum reproductive potential, while reproductive output in the large tank was about 50% down because of mild sperm limitation.

Previous investigations of storage of exogenous sperm by *D. listerianum* have documented intervals between sperm uptake and fertilization of up to one month (Bishop & Ryland 1991; Bishop & Sommerfeldt 1996; Bishop 1996), or have not included a time-scale (Burighel & Martinucci 1994*a*). The results of experiment 4 considerably extend the known maximum period of storage, with the last few embryos to be produced representing storage intervals of over seven weeks.

Models of fertilization success as a function of gamete dilution by turbulent diffusion (Denny 1988; Denny & Shibata 1989; Denny *et al.* 1992; Babcock *et al.* 1994; Levitan & Young 1995), incorporating equations for fertilization kinetics involving rates of gamete encounter (Rothschild & Swann 1951; Vogel *et al.* 1982; Levitan *et al.* 1991; Levitan 1993), have generated a considerable understanding of the problems of external fertilization in broadcast-spawning species. It is becoming apparent that conclusions from these models must be heavily modified when applied to brooding, suspension-feeding species. Mechanisms of internal fertilization, bringing male and female gametes into prolonged proximity, may substantially reduce the number of sperm per egg necessary to ensure a high level of fertilization. The potential to collect sperm over a period of time may render exact synchronization of gamete maturation/spawning relatively unimportant. A pumped inhalant current may draw sperm in from a large surrounding volume, having an effect superficially analogous to a chemical sperm attractant, but potentially functioning over a much larger area. In experiment 1, one hour exposures to sperm concentrations with an initial value of approximately 10 sperm ml^{-1} produced an appreciable number of fertilizations in *D. listerianum*. Fertilization was negligible at similar concentrations in several studies of external fertilization, many of which involved longer exposure periods in (i) sea urchins (Lillie 1915; Rothschild & Swann 1951; Vogel *et al.* 1982; Pennington 1985; Levitan *et al.* 1991; Levitan 1993; Levitan & Young 1995; Mead & Denny 1995); and (ii) other invertebrates (Brown & Knouse 1973; Oliver & Babcock 1992; André & Lindegarth 1995; Coma & Lasker 1997). However, Benzie & Dixon (1994) reported fertilization success close to 100% at 50 sperm ml^{-1} in the starfish *Acanthaster planci*, given fresh sperm early in the breeding season and a high sperm/egg ratio; the kinetics of external fertilization in the starfish evidently differed substantially from those documented in sea urchins.

In broadcast-spawning species, large body size, with the concomitant production of very large quantities of sperm, may reduce the effects of dilution and enable fertilization at relatively great distances from the source male (Babcock & Mundy 1992; Babcock *et al.* 1994). A small ramet of *D. listerianum* like that used in experiment 2 has a dry weight of under 2 mg, and the number of sperm produced must be relatively small. The ability of the ascidian to mate at considerable distances suggests that the importance of direct dilution effects may be reduced in active suspension feeders with fertilization of retained eggs. Nevertheless, body size (in the context of *D. listerianum*, the number of mature zooids), and thus sperm output, could be

important in relation to competition for fertilizations with other sperm sources (Yund & McCartney 1994; Yund 1995; McCartney 1997). In addition to the direct effects of progressive dilution of sperm with distance, fair raffle-type competition from more locally derived, and thus more abundant, sperm may reduce further the rate of fertilization by distant mates (Yund & McCartney 1994; Yund 1995). However, as argued by Yund (1995), this pattern of attenuation could be partially offset in populations with natural genetic structure (rather than in experimentally transplanted arrays of specimens lacking kin structure) by differentially increased success of remote mates unrelated to the focal individual (Grosberg 1987, 1991; Rigney *et al.* 1993).

The emerging picture of *D. listerianum* is, therefore, of an organism releasing rapidly dispersing wisps of long-lived, and possibly quiescent, sperm. Accumulations of sperm in the female reproductive tract in both wild (Burighel *et al.* 1986; Burighel & Martinucci 1994*a*) and cultured (Bishop & Sommerfeldt 1996; Bishop 1996) specimens represent a re-concentration of diluted sperm from water processed by the animal. Accordingly, as present results indicate, fertilization success does not depend on the interception of a cloud of gametes recently released by a near neighbour: sperm can apparently be taken up after they have undergone considerable dilution. They may then be stored adjacent to the oocytes for several weeks prior to fertilization. The individual zooids of *D. listerianum* have relatively low fecundity, commonly producing single zygotes at intervals of 7–12 days in culture (Bishop & Ryland 1991). A very modest mean capture rate of compatible sperm will therefore satisfy the zooid's needs for outcrossing. (Similar comments would apply to ovicellate cheilostome bryozoans, in which zooids typically brood a succession of single embryos: Reed 1991). Taking all these considerations together, it seems improbable that sperm limitation is an acute problem in *D. listerianum*, or in other sessile aquatic invertebrates with comparable reproductive patterns, as it may be in those that release both eggs and sperm into the water. The relatively high level of 'background fertilizations' in experimental open-water arrays of *Botrylluss schlosseri* reported by Yund & McCartney (1994) may not necessarily indicate limited dilution of sperm from natural sources as postulated by Levitan (1995) but, as suggested by Yund (1995), might reflect the efficient extraction of very dilute native sperm from seawater.

The live-animal stages of these experiments were conducted at the Port Erin Marine Laboratory, University of Liverpool, where the author was in receipt of a NERC fellowship. I thank David Pennington for the use of the large tank, and Richard Miller for advice on handling sperm. Andrew Pemberton provided very helpful comments on the manuscript, as did Philip Yund and three anonymous referees.

REFERENCES

André, C. & Lindegarth, M. 1995 Fertilization efficiency and gamete viability of a sessile, free-spawning bivalve, *Cerastoderma edule*. *Ophelia* **43**, 215–227.

Babcock, R. C. & Mundy, C. N. 1992 Reproductive biology, spawning and field fertilization rates of *Acanthaster planci*. *Austr. J. Mar. Freshwater Res.* **43**, 525–534.

Babcock, R. C., Mundy, C. N. & Whitehead, D. 1994 Sperm diffusion models and *in situ* confirmation of long-distance fertilization in the free-spawning asteroid *Acanthaster planci*. *Biol. Bull. Mar. Biol. Lab. Woods Hole* **186**, 17–28.

Benzie, J. A. H. & Dixon, P. 1994 The effects of sperm concentration, sperm:egg ratio, and gamete age on fertilization success in crown-of-thorns starfish (*Acanthaster planci*) in the laboratory. *Biol. Bull. Mar. Biol. Lab. Woods Hole* **186**, 139–152.

Bishop, J. D. D. 1996 Female control of paternity in the internally fertilizing compound ascidian *Diplosoma listerianum*. I. Autoradiographic investigation of sperm movements in the female reproductive tract. *Proc. R. Soc. Lond.* B **263**, 369–376.

Bishop, J. D. D. & Ryland, J. S. 1991 Storage of exogenous sperm by the compound ascidian *Diplosoma listerianum*. *Mar. Biol.* **108**, 111–118.

Bishop, J. D. D. & Ryland, J. S. 1993 Enzyme electrophoretic evidence for the prevalence of outcrossing in the hermaphroditic brooding ascidian *Dendrodoa grossularia* (Chordata, Urochordata). *J. Exp. Mar. Biol. Ecol.* **168**, 149–165.

Bishop, J. D. D. & Sommerfeldt, A. D. 1996 Autoradiographic investigation of uptake and storage of exogenous sperm by the ovary of the compound ascidian *Diplosoma listerianum*. *Mar. Biol.* **125**, 663–670.

Bishop, J. D. D., Jones, C. S. & Noble, L. R. 1996 Female control of paternity in the internally fertilizing compound ascidian *Diplosoma listerianum*. II. Investigation of male mating success using RAPD markers. *Proc. R. Soc. Lond.* B **263**, 401–407.

Bolton, T. F. & Havenhand, J. N. 1996 Chemical mediation of sperm activity and longevity in the solitary ascidians *Ciona intestinalis* and *Ascidiella aspersa*. *Biol. Bull. Mar. Biol. Lab. Woods Hole* **190**, 329–335.

Brazeau, D. A. & Lasker, H. R. 1992 Reproductive success in the Caribbean octocoral *Briareum asbestinum*. *Mar. Biol.* **114**, 157–163.

Brown, G. G. & Knouse, J. R. 1973 Effects of sperm concentration, sperm aging, and other variables on fertilization in the horse-shoe crab, *Limulus polyphemus* L. *Biol. Bull. Mar. Biol. Lab. Woods Hole* **144**, 462–470.

Burighel, P. & Martinucci, G. B. 1994*a* Sexual reproduction in the compound ascidian *Diplosoma listerianum* (Tunicata). I. Metamorphosis, storage and phagocytosis of sperm in female duct. *Mar. Biol.* **118**, 489–498.

Burighel, P. & Martinucci, G. B. 1994*b* Sexual reproduction in the compound ascidian *Diplosoma listerianum* (Tunicata). II. Sperm penetration through ovary wall and evidence of internal fertilization. *Mar. Biol.* **118**, 499–510.

Burighel, P., Martinucci, G. B. & Magri, F. 1985 Unusual structures in the spermatozoa of the ascidians *Lissoclinum perforatum* and *Diplosoma listerianum* (Didemnidae). *Cell Tissue Res.* **241**, 513–521.

Burighel, P., Martinucci, G. B. & Zaniolo, G. 1986 Ovulation in the ovoviviparous ascidian, *Diplosoma listerianum*. *Acta Embryol. Morphol. Exp.* **7**, 102–103.

Coma, R. & Lasker, H. R. 1997 Small-scale heterogeneity of fertilization success in a broadcast spawning octocoral. *J. Exp. Mar. Biol. Ecol.* **214**, 107–120.

Craig, S. F., Slobodkin, L. B., Wray, G. A. & Biermann, C. H. 1997 The 'paradox' of polyembryony: a review of the cases and a hypothesis for its evolution. *Evol. Ecol.* **11**, 127–143.

Denny, M. W. 1988 *Biology and the mechanics of the wave-swept environment*. Princeton University Press.

Denny, M. W. & Shibata, M. F. 1989 Consequences of surf-zone turbulence for settlement and external fertilization. *Am. Nat.* **134**, 859–889.

Denny, M., Dairiki, J. & Distefano, S. 1992 Biological consequences of topography on wave-swept rocky shores. I. Enhancement of external fertilization. *Biol. Bull. Mar. Biol. Lab. Woods Hole* **183**, 220–232.

Grosberg, R. K. 1987 Limited dispersal and proximity-dependent mating success in the colonial ascidian *Botryllus schlosseri*. *Evolution* **41**, 372–384.

Grosberg, R. K. 1991 Sperm-mediated gene flow and the genetic structure of a population of the colonial ascidian *Botryllus schlosseri*. *Evolution* **45**, 130–142.

Harumi, T., De Santis, R., Pinto, M. R. & Suzuki, N. 1990 Phospholipid utilization in ascidian *Ciona intestinalis* spermatozoa during swimming. *Comp. Biochem. Physiol.* **96A**, 263–265.

Havenhand, J. N. 1991 Fertilisation and the potential for dispersal of gametes and larvae in the solitary ascidian *Ascidia mentula* Müller. *Ophelia* **33**, 1–15.

Lasker, H. R., Brazeau, D. A., Calderon, J., Coffroth, M. A., Coma, R. & Kim, K. 1996 *In situ* rates of fertilization among broadcast spawning gorgonian corals. *Biol. Bull. Mar. Biol. Lab. Woods Hole* **190**, 45–55.

Levitan, D. R. 1991 Influence of body size and population density on fertilization success and reproductive output in a free-spawning invertebrate. *Biol. Bull. Mar. Biol. Lab. Woods Hole* **181**, 261–268.

Levitan, D. R. 1993 The importance of sperm limitation to the evolution of egg size in marine invertebrates. *Am. Nat.* **141**, 517–536.

Levitan, D. R. 1995 The ecology of fertilization in free-spawning invertebrates. In *Ecology of marine invertebrate larvae* (ed. L. McEdward), pp. 123–156. Boca Raton: CRC Press.

Levitan, D. R. 1996*a* Effects of gamete traits on fertilization in the sea and the evolution of sexual dimorphism. *Nature* **382**, 153–155.

Levitan, D. R. 1996*b* Predicting optimal and unique egg sizes in free-spawning marine invertebrates. *Am. Nat.* **148**, 174–188.

Levitan, D. R. & Petersen, C. 1995 Sperm limitation in the sea. *Trends Ecol. Evol.* **10**, 228–231.

Levitan, D. R., Sewell, M. A. & Chia, F.-S. 1991 Kinetics of fertilization in the sea urchin *Strongylocentrotus franciscanus*: interaction of gamete dilution, age, and contact time. *Biol. Bull. Mar. Biol. Lab. Woods Hole* **181**, 371–378.

Levitan, D. R., Sewell, M. A. & Chia, F.-S. 1992 How distribution and abundance influence fertilisation success in the sea urchin *Strongylocentrotus franciscanus*. *Ecology* **73**, 248–254.

Levitan, D. R. & Young, C. M. 1995 Reproductive success in large populations: empirical measures and theoretical predictions of fertilization in the sea biscuit *Clypeaster rosaceus*. *J. Exp. Mar. Biol. Ecol.* **190**, 221–241.

Lillie, F. R. 1915 Studies of fertilization. VII. Analysis of variations in the fertilizing power of sperm suspensions of *Arbacia*. *Biol. Bull. Mar. Biol. Lab. Woods Hole* **28**, 229–251.

McCartney, M. A. 1997 Sex allocation and male fitness gain in a colonial, hermaphroditic marine invertebrate. *Evolution* **51**, 127–140.

Mead, K. S. & Denny, M. W. 1995 The effects of hydrodynamic shear stress on fertilization and early development of the purple sea urchin *Strongylocentrotus purpuratus*. *Biol. Bull. Mar. Biol. Lab. Woods Hole* **188**, 46–56.

Miller, R. L. 1982 Sperm chemotaxis in ascidians. *Am. Zool.* **22**, 827–840.

Miller, R. L. 1994 Mechanisms for enhancing fertilization success in two species of free-spawning invertebrates with internal fertilization. In *Reproduction and development of marine invertebrates* (ed. W. H. Wilson Jr, S. A. Stricker & G. L. Shinn), pp. 106–117. Baltimore and London: Johns Hopkins University Press.

Miller, R. L. 1996 Chemosensory phenomena during sexual interactions in gelatinous zooplankton. *Mar. Freshwater Behav. Physiol.* **27**, 163–173.

Minganti, A. 1951 Esperienze sulle fertilizine nelle ascidie. *Pubbl. Staz. Zool. Napoli* **22**, 58–65.

Morisawa, M. 1994 Cell signaling mechanisms for sperm motility. *Zool. Sci.* **11**, 647–662.

Oliver, J. & Babcock, R. 1992 Aspects of the fertilization ecology of broadcast spawning corals: sperm dilution effects and *in situ* measurements of fertilization. *Biol. Bull. Mar. Biol. Lab. Woods Hole* **183**, 409–417.

Pennington, J. T. 1985 The ecology of fertilization of echinoid eggs: the consequences of sperm dilution, adult aggregation, and synchronous spawning. *Biol. Bull. Mar. Biol. Lab. Woods Hole* **169**, 417–430.

Petersen, C. W., Warner, R. R., Cohen, S., Hess, H. C. & Sewell, A. T. 1992 Variable pelagic fertilization success: implications for mate choice and spatial patterns of mating. *Ecology* **73**, 391–401.

Reed, C. G. 1991 Chapter 3. Bryozoa. In *Reproduction of marine invertebrates. Volume VI A. Echinoderms and lophophorates*. (ed. A. C. Giese, J. S. Pearse & V. B. Pearse), pp. 85–245. Pacific Grove, CA: The Boxwood Press.

Rigney, L. P., Thomson, J. D., Cruzan, M. B. & Brunet, J. 1993 Differential success of pollen donors in a self-compatible lily. *Evolution* **47**, 915–924.

Rothschild, L. & Swann, M. M. 1951 The fertilization reaction in the sea urchin. The probability of a successful sperm-egg collision. *J. Exp. Biol.* **28**, 403–416.

Ryland, J. S. & Bishop, J. D. D. 1990 Prevalence of cross-fertilization in the hermaphroditic compound ascidian *Diplosoma listerianum*. *Mar. Ecol. Prog. Ser.* **61**, 125–132.

Ryland, J. S. & Bishop, J. D. D. 1993 Internal fertilisation in hermaphoditic colonial invertebrates. *Oceanogr. Mar. Biol.* **31**, 445–477.

Spärck, R. 1927 Studies on the biology of the oyster (*Ostrea edulis*). IV. On fluctuations in the oyster stock in the Limfjord. *Rep. Dan. Biol. Stn* **33**, 60–65.

Suquet, M., Billard, R., Cosson, J., Dorange, G., Chauvaud, L., Mugnier, C. & Fauvel, C. 1994. Sperm features in turbot (*Scophthalmus maximus*): a comparison with other freshwater and marine fish species. *Aquat. Living Resources* **7**, 283–294.

Temkin, M. H. 1991 *Fertilization in the gymnolaemate Bryozoa*. Ph.D. thesis, University of Southern California.

Temkin, M. H. 1996 Comparative fertilization biology of gymnolaemate bryozoans. *Mar. Biol.* **127**, 329–339.

Trippel, E. A. & Morgan, M. J. 1994 Sperm longevity in Atlantic cod (*Gadus morhua*). *Copeia* **1994**, 1025–1029.

Tuzet, O., Bogoraze, D. & Lafargue, F. 1972 Recherches ultrastructurales sur la spermiogenèse de *Diplosoma listerianum* (Milne-Edwards, 1841) et *Lissoclinum pseudoleptoclinum* (Von Drasche, 1883) (ascidies composées, aplousobranches). *Ann. Sci. Nat., Zool., 12e Série* **14**, 177–190.

Vogel, H., Czihak, G., Chang, P. & Wolf, W. 1982 Fertilization kinetics of sea urchin eggs. *Math. Biosci.* **58**, 189–216.

Yoshida, M., Inaba, K. & Morisawa, M. 1993 Sperm chemotaxis during the process of fertilization in the ascidians *Ciona savignyi* and *Ciona intestinalis*. *Devl Biol.* **157**, 497–506.

Yund, P. O. 1990 An *in situ* measurement of sperm dispersal in a colonial marine hydroid. *J. Exp. Zool.* **253**, 102–106.

Yund, P. O. 1995 Gene flow via the dispersal of fertilizing sperm in a colonial ascidian (*Botryllus schlosseri*): the effect of male density. *Mar. Biol.* **122**, 649–654.

Yund, P. O. & McCartney, M. A. 1994 Male reproductive success in sessile invertebrates: competition for fertilizations. *Ecology* **75**, 2151–2167.