Vol. 215: 191–200, 2001 | MARINE ECOLOGY PROGRESS SERIES
Mar Ecol Prog Ser | Published May 31

Effect of sperm concentration and sperm ageing on fertilisation success in the Antarctic soft-shelled clam *Laternula elliptica* and the Antarctic limpet *Nacella concinna*

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ABSTRACT: Sperm concentration and sperm ageing effects on fertilisation success were evaluated in the laboratory in the free-spawning Antarctic soft-shelled clam *Laternula elliptica* and Antarctic limpet *Nacella concinna*. Fertilisation success was highly dependent on sperm concentration. Highest levels of fertilisation success were consistently replicated at ~10^7 sperm ml^{-1} for *L. elliptica*, and ~10^6 to 10^8 sperm ml^{-1} for *N. concinna*. However, both species exhibited extremely low fertilisation rates at concentrations <10^6 sperm ml^{-1}. At sperm concentrations >10^6 sperm ml^{-1} *N. concinna* displayed a rapid increase in abnormally developing larvae which, along with only a small decline in total fertilisation success above 10^8 sperm ml^{-1}, was taken to indicate polyspermy. A small increase in abnormal development followed by a rapid decline in fertilisation success at high sperm concentrations (>10^7 sperm ml^{-1}) for *L. elliptica* was attributed to oxygen depletion. Using the optimum sperm concentration found for fertilisation success, spermatozoa were capable of fertilising fresh ova for >90 h in *L. elliptica*, and ~65 h in *N. concinna*. The sperm concentrations required for fertilisation success and sperm longevities reported here are at least an order of magnitude greater than those reported for nearshore temperate molluscs. Our data strongly suggest that the specific reproductive behaviour and timing of spawning activities displayed by these Antarctic molluscs is vital to enhance fertilisation success in the polar marine environment.

KEY WORDS: Fertilisation success · Antarctica · Reproduction · Free-spawning · Sperm concentration · Sperm age · *Laternula elliptica* · *Nacella concinna*

INTRODUCTION

Successful fertilisation of eggs is a key element of maximising fitness in animals. To understand fertilisation success in free-spawning marine invertebrates or perhaps, more importantly, fertilisation failure, a detailed understanding of the factors affecting this process is crucial. These factors include the distribution, density and behaviour of reproductively active individuals, the density of sperm required to fertilise eggs, and the longevity and behaviour of the gametes. There are not only complex interactions between the above factors, but also with the physical properties and dynamics of the fluid medium. The relative importance of each factor to fertilisation success remains obscure (Levitan 1995).

Recent research has focussed on nearshore temperate and tropical free-spawning marine invertebrates, particularly those amenable to field and laboratory manipulation (see reviews by Levitan 1995, Levitan & Petersen 1995, Yund 2000). These include echinoderms, polychaetes and corals (Pennington 1985, Levitan et al. 1992, Oliver & Babcock 1992, Benzie & Dixon 1994, Lasker et al. 1996, Williams et al. 1997) and com-

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mercial molluscan species, including abalone *Haliotis* spp. (Clavier 1992, Mill & McCormick 1992, Encena et al. 1998, Babcock & Keesing 1999, Baker & Tyler 2001), oyster *Crassostrea gigas*, Manilla clam *Tapes decussatus*, limpet *Patella vulgata* (M. Baker unpubl. data), cockle *Cerastoderma edule* (André & Lindegarth 1995), mussel *Mytilus* spp. (Sprung & Bayne 1984), and scallop *Chlamys bifrons* and *C. asperrima* (Styan 1998a).

Successful fertilisation is possible over a wide range of sperm concentrations (~10^3 to 10^6 sperm ml^{-1}) when the animals are well within the boundaries of their physical environmental tolerances. However, fertilisation rates decrease rapidly as sperm become diluted in the water column and less potent over time (Pennington 1985, Levitan et al. 1992). To counteract dilution and gamete ageing effects, and to maximise the chances of fertilisation, free-spawning marine animals have developed strategies to increase the probabilities of sperm:egg contact soon after release. For species that fertilise externally, strategies include living in close proximity or forming aggregations and synchronising spawning activities as well as spawning in low to moderate flow regimes, in shallow water or confined areas (Levitan 1995).

Many of the most common and ubiquitous nearshore marine invertebrate species in Antarctica are free-spawning and produce pelagic larvae despite living at low temperatures (ranging between +2 and −1.8°C) (Clarke 1992, Pearse 1994, Pearse & Bosch 1994, Stanwell-Smith et al. 1998). Antarctic free-spawners also display behavioural strategies of aggregating and synchronous spawning (Picken & Allan 1983, Stanwell-Smith & Clarke 1998). However, polar studies on the factors affecting fertilisation success and the importance of their behavioural strategies have not previously been reported.

For Antarctic marine fauna, low seawater temperatures affect the physical properties of the water by increasing gas solubility, viscosity and density, and by lowering the pH. Physiological and biochemical processes are also slowed (Pörtner & Playle 1998). Antarctic marine animals characteristically have deferred maturation, slower gametogenic cycles (Clarke 1988), lower fecundity (Clarke 1979), slowed embryonic development and greatly extended larval periods (Clarke 1982, Bosch & Pearse 1990, Hoegh-Guldberg & Pearse 1995, Stanwell-Smith & Peck 1998, S. Hain unpubl.) compared to temperate species. The low temperature also affects the ability of the gametes to function effectively in the water column. For example, sperm of the Antarctic echinoid *Sterechinus neumayeri* binds quickly to eggs, but the timing of the early fertilisation event is significantly slower than for temperate species (Stokes et al. 1996). Reduced temperatures also lower sperm activity and decrease fertilisation success (Greenwood & Bennett 1981) directly via increased viscosity which restricts sperm motility (Vogel 1994). Whether these factors have a positive or negative effect on fertilisation success in Antarctic marine fauna has not been tested. Many physical factors affecting Antarctic species also apply to the deep sea and our data could be pertinent to evaluations of fertilisation success in abyssal fauna.

In the present study sperm dilution and sperm ageing experiments were conducted that are comparable with those used for temperate and tropical free-spawners. The species chosen for this study were the infaunal soft-shelled clam *Laternula elliptica* and the patellid limpet *Nacella concinna.* Both free-spawn directly into the water column and are common around Antarctica and the sub-Antarctic islands. *L. elliptica* inhabits soft sediments in water depths of 3 to 320 m (Lan 1994, Sahade et al. 1998), where it can form dense beds of up to 200 ind. m^{-2} (Wägele & Brito 1990). They can burrow deep (>50 cm) (Hardy 1972) and feed on suspended phytoplankton using extended siphons (Ahn 1994). *L. elliptica* may live for >20 yr (Brey & Mackensen 1997) and reach shell lengths of ~110 mm (Urban & Mercuri 1998). It is a simultaneous hermaphrodite with external fertilisation, producing lecithotrophic embryos which develop intracapsularly before hatching at an advanced juvenile stage to settle onto the sediments (Bosch & Pearse 1988). Spawning occurs in the austral summer, autumn and winter, sometimes in epidemic spawning events (Bosch & Pearse 1988, Urban & Mercuri 1998, Ahn et al. 2000, D. Powell unpubl. data, R. Luxmoore pers. comm.).

The limpet *Nacella concinna* does not 'home', but roams freely over hard and soft substrata, grazing on algae and diatoms from the littoral zone to depths of ~110 m (Picken 1980). It reportedly reaches shell lengths of ~41 mm in 21 yr and lives for ~60 yr (Shabica 1976, Picken 1980). *N. concinna* are dioecious, fertilise externally (Shabica 1976, Picken 1980) and produce pelagic larvae. However, reports are rare, and there is speculation on the trophic status of the larvae (Picken 1980, Stanwell-Smith & Clarke 1998). Unusually for a patellid limpet, *N. concinna* commonly forms spawning clusters or stacks of ~3 to 35 individuals for up to 7–10 d during the spring bloom period (Picken & Allan 1983, Stanwell-Smith & Clarke 1998).

MATERIAL AND METHODS

Study site and laboratory. Experiments were carried out between December 1998 and March 1999 at Rothera Research Station (67° 34′ 07″ S, 68° 07′ 30″ W), Adelaide Island, Antarctic Peninsula. All practical work

was conducted in a controlled temperature aquarium room held at a constant 2°C in a 12 h light/dark cycle. The flow-through aquarium system consisted of many large adjoining plastic tanks maintained at near ambient summer seawater temperatures (0 to 0.9°C).

Collection of animals. Adult *Laternula elliptica* were collected by SCUBA divers from the west side of the airstrip in North Cove (67° 33′ 40″ S, 68° 07′ 30″ W) at a depth of 14 m. Adult *Nacella concinna* were collected from the same site and near the wharf in South Cove (67° 34′ 17″ S, 68° 07′ 50″ W) at depths between 5 and 10 m. The animals were transported directly to the aquarium, where clams were held in the main aquarium whilst limpets were held in free-standing, 10 l aerated glass aquaria. The seawater in the glass aquaria was aerated using stone diffusers and changed twice daily to avoid the build up of waste products. Animals were allowed to acclimate for up to 1 wk prior to experimentation.

Collection of the gametes. Neither clams nor limpets could be induced to spawn by thermal shock, air shock or by chemical stimulus (serotonin). Gametes were, therefore, obtained by strip-spawning. For this procedure, clean pipettes, scalpels and glassware were used at each stage of the process to avoid cross contamination of the gametes.

Collection of Laternula elliptica gametes: Although hermaphrodite, for each experimental trial separate individuals were used as the 'female' and the 'male' to achieve cross-fertilisation. Eggs were collected first to allow them to acclimate and activate in the seawater. The valves of the clam were prised apart, and the female area of the gonad lining was carefully punctured to release the eggs. The eggs were collected in a sterile glass capillary and transferred to a glass beaker containing 100 ml USW (unfiltered seawater) at ~0°C. Sperm were collected from a puncture made at the rear of the gonad. As much 'dry' sperm as possible were collected using a sterile glass capillary and added to a glass finger bowl containing 10 ml USW at ~0°C. This created the concentrated sperm stock solution.

Collection of Nacella concinna gametes: Live limpets were sexed before commencing strip-spawning as the female pinkish/purple ovary was easy to distinguish from the male cream-coloured testis. The gonad was exposed by dissection, and the lining carefully punctured to release the eggs or sperm. Eggs were collected using a sterile glass capillary and put in a glass beaker containing 50 ml USW at ~0°C. The 'dry' sperm were added to a glass finger bowl containing 10 ml USW at ~0°C to create the sperm stock solution.

For both species, 5 ml of sperm stock solution was preserved with a few drops of 10% formalin to determine sperm concentration using a Neubauer haemocytometer. If the eggs were irregular in shape or size, or the spermatozoa were inactive or sluggish, fresh gametes were quickly stripped from a new individual.

Effects of sperm dilution on fertilisation success in *Laternula elliptica* and *Nacella concinna*. for each sperm dilution trial, 24 × 30 ml glass vials were inserted into pre-cut holes in a polystyrene sheet in 8 rows of 3. The sheet was floated in an aquarium tank throughout the trial so that all vials were exposed to the same conditions. A sperm dilution gradient was created in triplicate along the rows of vials by diluting the concentrated stock sperm suspension through a series of 10-fold dilutions giving a range of sperm dilutions from ~10^9 to ~10^1 sperm ml^{-1}. Each row, therefore, contained 3 replicate vials each with 9 ml of a sperm dilution; 1 ml of egg water was then added to each vial. To check for self-fertilisation, 3 control vials contained 1 ml of egg water and 9 ml USW. The vials were agitated on introduction of the gametes and at regular intervals to ensure good mixing. A culture of the remaining sperm and eggs from each pairing was made and held in an aerated 2 l beaker at ambient temperature (+0.5 to +0.9°C) as a further control. The seawater in the 2 l beakers was partially exchanged for clean seawater daily. The controls were checked twice daily. If self fertilisation, cross-contamination or poor development was evident in the control vials or cultures then the experiment was terminated. Each *N. concinna* and *L. elliptica* trial was run for 24 and 48 h respectively to allow fertilisation and cleavage to proceed to the 8 to 16 cell stage, when normal development, abnormal development or no fertilisation could be differentiated. The cleaving embryos were scored as abnormal if they exhibited morphological anomalies that would ultimately lead to a cessation of development (based on previous observations of larval development in the study species).

In preliminary trials spermatozoa were washed from the eggs 3 h after fertilisation, but this technique had adverse effects on fertilisation success and subsequent development. Therefore, all the sperm dilutions were held constant in the vials during the experimental period.

Sperm age experiments on *Laternula elliptica* and *Nacella concinna*. The sperm concentrations which maximized fertilisation success for *L. elliptica* (1×10^7 sperm ml^{-1}) and *N. concinna* (3.6×10^6 sperm ml^{-1}) determined from the sperm dilution experiments were used in the sperm age experiments. In the *N. concinna* trial, fresh sperm and eggs were obtained from naturally spawning limpets in the aquarium. The time of natural sperm spawning (*N. concinna*) or strip-spawning (*L. elliptica*) was recorded as sperm age 0 h.

For the sperm age experiments, 1 ml of egg water (created as per sperm dilution experiments) from Female 1 was added immediately to 3 replicate vials each containing 9 ml of sperm solution. The remaining

egg water was held as a control for 6 h to check for fitness or cross-contamination before being discarded. The ageing sperm dilution was held in a 500 ml glass beaker at ambient seawater temperature until the next sampling period. After 12 h, the process was repeated with eggs stripped from another female, and this process was repeated at regular intervals until no fertilisation of freshly stripped eggs was obtained. The eggs in the vials were left to develop for 24 or 48 h as in the previous experimental protocol. The experiment was terminated when no fertilisation had occurred in the most recently prepared vials after 12 h.

Measurements of fertilisation success. At the end of each experimental trial, 1 ml of eggs from each vial was pipetted into a counting cell and viewed under a stereomicroscope (×40). The numbers of 'normally developing', 'abnormally developing' and 'unfertilised' eggs were scored (n = 50).

Measurement of oxygen content during the *Laternula elliptica* sperm dilution experiment. A couloximeter was used to measure the oxygen content of the water in the culture vials containing sperm concentrations of between 10^8 and 10^4 sperm ml^{-1} after the 48 h culture period. During 1 trial for *L. elliptica* (Trial 5) replicated 25 µl water samples were taken from the vials using a SGE gas-tight syringe. Each water sample was injected into the fuel-cell-based couloximeter, which measured the oxygen content to ±1% (Peck & Uglow 1990). Experimental controls were obtained from replicated 25 µl samples taken from a vial containing 10 ml USW, also left for 48 h.

***In situ* sampling of *Nacella concinna* spawning sperm.** To assess the quantity of sperm ejected by a limpet during a natural spawning event, ripe males in the aquarium were encouraged to spawn by gently tapping the shell. Spawning males were then held in 20 ml seawater until gamete release was complete. In the field, the sperm released in 1 pulse from an individual male was sampled using a 20 ml syringe held at the expulsion site. The sperm was preserved, and sperm counts made using a haemocytometer.

Statistical analysis. All calculations and statistical manipulations were made using Lotus 123 in Windows 97 and plotted using Sigma Plot 5.0.

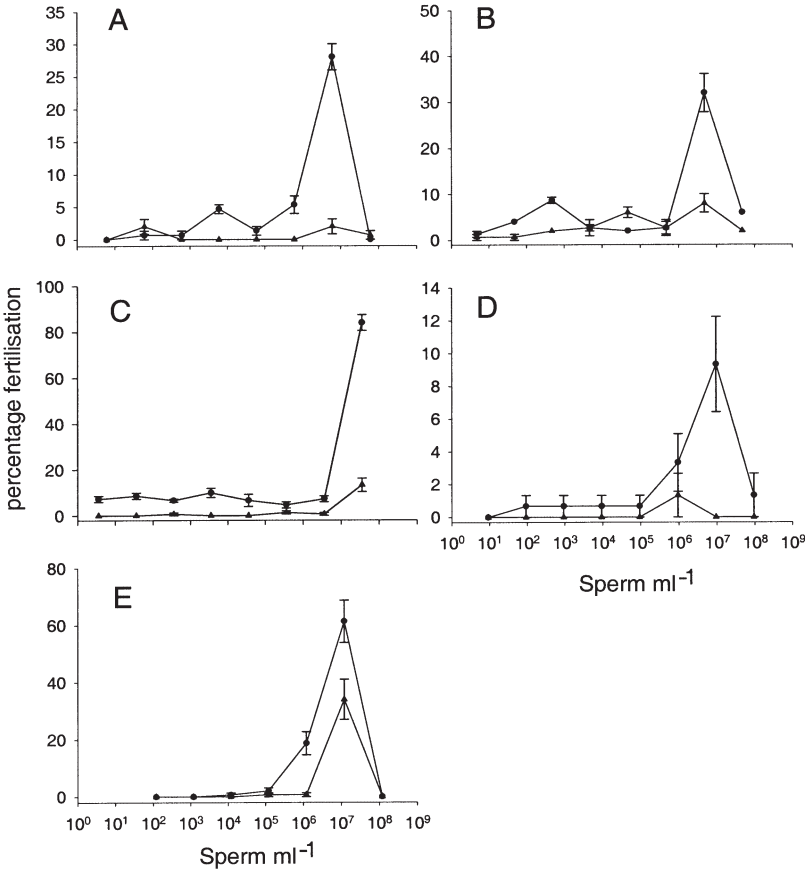

Fig. 1. *Laternula elliptica*. Individual replicates of the percentage of normal (●) and abnormal (▲) developing embryos produced after fertilisation at different sperm concentrations. Data are the mean ± SE of 3 replicates

RESULTS

Effects of sperm dilution on fertilisation success in *Laternula elliptica*

There was great variability in fertilisation success between the different male/female pairings in the 5 experimental trials (maxima ranging from to 9 to 97%) (Fig. 1A to E). However, even though maximum fertilisation success in 3 of the 5 trials was <50% (Fig. 1 A,B,D), all 5 trials showed a distinct peak between 5×10^6 and 4×10^7 sperm ml^{-1} and a sharp drop in the percentage fertilised above and below these values. A few abnormally developing embryos were also present in all the trials, the peak in abnormalities coinciding with the peak for total fertilisation success.

Effects of sperm dilution on fertilisation success in *Nacella concinna*

As with *Laternula elliptica*, there was great variability in fertilisation success between different male/female pairings with maximum fertilisation rates of ~12, 80 and 98% in each trial (Fig. 2A to C).

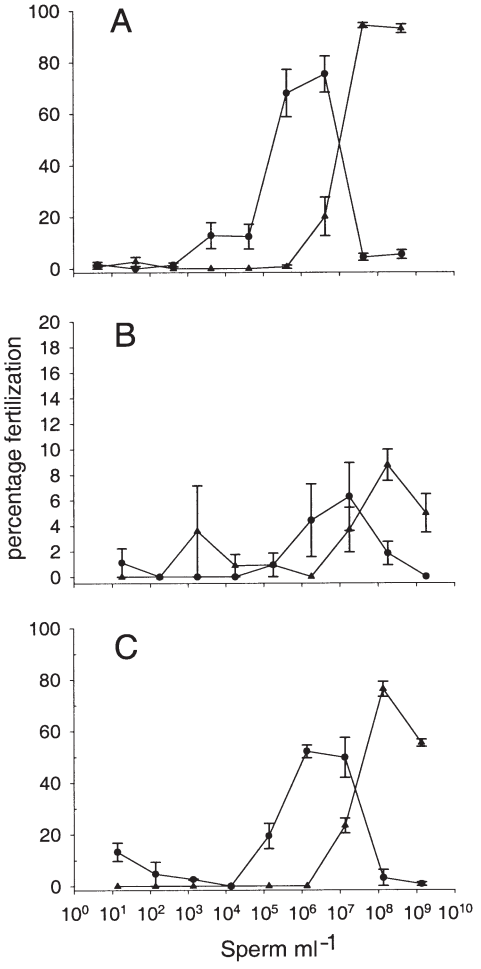

Fig. 2. *Nacella concinna*. Individual replicates of the percentage of normal (●) and abnormal (▲) developing embryos produced after fertilisation at different sperm concentrations. Data are the mean ± SE of 3 replicates

Despite this, all 3 trials showed the same pattern, with a noticeable increase in fertilisation success from 10^6 to 10^8 sperm ml^{-1}. In all trials, fertilisation success declined at concentrations >10^8 sperm ml^{-1}. In all 3 trials, a sharp rise in the number of abnormally developing embryos occurred above 10^6 sperm ml^{-1}, with a decrease in normal development occurring beyond 10^7 sperm ml^{-1} (Fig. 2A,C). The sperm concentration that produced the highest proportion of normally developing embryos was between 4×10^6 and 2×10^7 sperm ml^{-1}.

Effect of sperm ageing on fertilisation success in *Laternula elliptica*

Fertilisation of fresh eggs was still possible using sperm (at a concentration of 1×10^7 sperm ml^{-1}) aged 90+ h. For the first 37 h, total fertilisation success was high at 75 to 80% (Fig. 3). After 40 h, fertilisation success dropped to <25 % and continued to fall, reaching 0 after 110 h. The standard error bars for each individual female are small, indicating good replication of the data at each time interval. The sharp decline in fertilisation success at 40 to 50 h may therefore be indicative of variability in the quality of eggs between females.

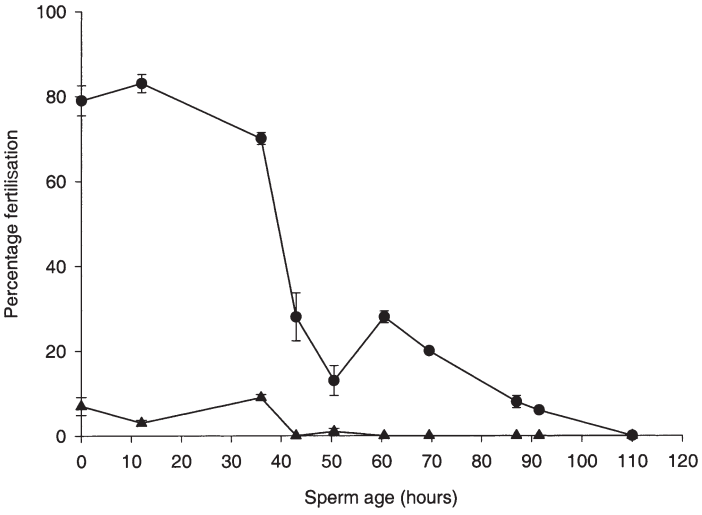

Fig. 3. *Laternula elliptica*. Effect of sperm age on the percentage of normal (●) and abnormal (▲) developing embryos (sperm concentration = 10^7 sperm ml^{-1}). Data are the mean ± SE of 3 replicates

Effect of sperm ageing on fertilisation success in *Nacella concinna*

Preliminary trials using stripped sperm revealed that fertilisation was possible up to 70 h after release (Powell unpubl. data). A similar result was achieved using naturally spawned sperm, with fertilisation of fresh eggs still possible using sperm 65 h after release (Fig. 4). The greatest fertilisation success (~80%) was achieved when the spermatozoa were freshly spawned. Fertilisation success declined rapidly as the sperm aged, reaching values below 50% after 25 h. There was little variation between fertilisation success in different females. However, for sperm ages of 0 to 30 h, there was considerable variation between percentage of normal versus abnormal development, indicating differences in egg quality between the different females used.

Oxygen content of the culture water in the *Laternula elliptica* sperm dilution experiments

Oxygen content in cultures from Trial 5 of the *Laternula elliptica* sperm dilution experiment declined

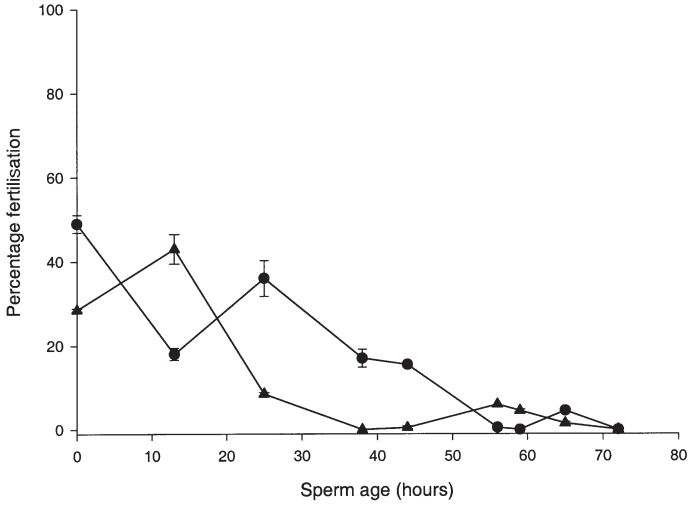

Fig. 4. *Nacella concinna*. Effect of sperm age on the percentage of normal (●) and abnormal (▲) developing embryos (sperm concentration = 3.16×10^6 sperm ml^{-1}). Data are the mean ± SE of 3 replicates

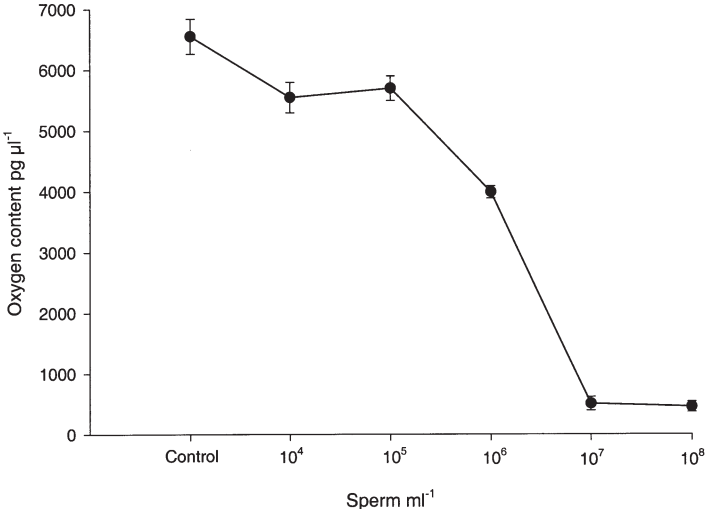

Fig. 5. *Laternula elliptica*. Oxygen content (pg µl^{-1}) of culture water in relation to sperm density (no. ml^{-1}). Measurements were made after cultures had run for 48 h in the experimental vials. Our data were only collected from 1 trial (Trial 5). Data are the mean ± SE of 3 replicates

Table 1. *Nacella concinna.* Total sperm concentration spawned naturally by *N. concinna* held in the aquarium

	Total sperm (ml^{-1})
Male 1	9.15×10^5
Male 2	1.88×10^6
Male 3	5.40×10^6
Male 4	3.60×10^6

steadily with increasing sperm concentration, indicating increased oxygen consumption by the gametes (Fig. 5).

Sperm concentrations in naturally spawning *Nacella concinna*

Four spawning male limpets were sampled in the aquarium and produced between 9.2×10^5 and 5.6×10^6 sperm ml^{-1} (Table 1). The time taken for each individual to complete spawning was variable, and they produced intermittent pulses of sperm rather than 1 continuous release. Spawning times were not rigorously recorded, but ranged from ~10 min to several hours, with the smaller animals completing their spawning more rapidly (Powell pers. obs.).

DISCUSSION

Fertilisation success in *Laternula elliptica* and *Nacella concinna* was highly dependent on sperm concentration. The range of concentration needed to optimize fertilisation in these animals is extremely narrow, especially for *L. elliptica*. The greatest numbers of fertilised embryos consistently occurred at sperm concentrations of ~5×10^6 to 5×10^7 sperm ml^{-1} for *L. elliptica* and 4×10^6 to 1×10^7 sperm ml^{-1} for *N. concinna*, with marked reductions in fertilisation success above and below these concentrations. These results differ from those reported for nearshore temperate and tropical free-spawning marine invertebrates in that, although the same percentages of fertilisation success are achieved in the Antarctic, this is only so over a much smaller range of sperm concentrations and only at concentrations 1 to 2 orders of magnitude higher. Temperate and tropical species have optimum ranges for fertilisation success between 10^3 and 10^6 sperm ml^{-1} (Clavier 1992, Clotteau & Dube 1993, Williams et al. 1997, Encena et al. 1998, Babcock & Keesing 1999, Baker & Tyler 2001). Good rates of fertilisation are still achievable at sperm concentrations as low as 10^2 sperm ml^{-1} (Pennington 1985, Levitan et al. 1991, M. Baker unpubl. data). Only a few studies have shown temperate and tropical species reaching peaks in fertilisation success at sperm concentrations as high as 10^7 to 10^8 sperm ml^{-1} (Levitan et al. [1991] for the sea urchin, *Strongylocentrotus franciscanus*; Fong et al. [1995] for the freshwater zebra mussel *Dreissena polymorpha*; Williams & Bentley [in press] for the temperate polychaete *Nereis virens*). However, it is not clear in these studies if the embryos were developing normally after being subjected to such high sperm concentrations.

It is possible that taxonomic differences between the species studied in Antarctica and tropical/temperate

latitudes could account for the high sperm concentrations needed to optimize fertilisation success. However, it is more likely that the depressed levels of fertilisation success in both Antarctic species below 10^6 sperm ml^{-1} are caused by a combination of factors such as lowered sperm activity (Greenwood & Bennett 1981), lowered pH (Oliver & Babcock 1992) and increased viscosity and density in the low temperature conditions. The requirement for very high sperm concentrations to achieve fertilisation success in *Laternula elliptica* and *Nacella concinna* may indicate a higher energetic cost to reproduction at low temperatures not previously considered for Antarctic marine fauna. Moreover, the data suggest that specific spawning behaviours and timing of spawning are vital for these Antarctic molluscs to achieve high levels of fertilisation success in the field, and to counteract any enhanced energetic costs. For example, if *L. elliptica* and *N. concinna* require a minimum of 10^6 sperm ml^{-1} for high fertilisation success, then success would be impaired by any significant water movement, even if the spawning individuals were separated by only a metre or so. Such animals therefore require specific behavioural strategies to counteract water dilution effects.

Synchronous release of large quantities of gametes enhances success by increasing the chances of sperm-egg contact, and cross-fertilisation for hermaphroditic species (Levitan 1995). *Laternula elliptica* populations occur in dense beds in soft sediments and have been observed spawning en masse late in the austral summer and autumn (Bosch & Pearse 1988) and in negligible water flow under fast ice in the winter (R. Luxmoore pers. comm.). In the winter event, the released sperm reduced visibility from 25 m to just 1 m for about 1 wk, the eggs becoming visible around the siphons nearly 1 wk later. The presence of fast ice could effectively reduce the volume of water around the spawning population, thereby increasing the concentrations of gametes around the animals. Gravid *Nacella concinna* have the unusual behavioural strategy of congregating in stacks of randomly sexed individuals for spawning purposes (Picken & Allan 1983). The close contact between males and females reduces the distance between sperm and eggs. This strategy may be necessary because the optimum range of sperm concentrations found in the sperm dilution experiments were of a similar order of magnitude to the maximum concentrations released by naturally spawning individuals in the aquarium and in the field. Opportunistic observations of the behaviour of *N. concinna* gametes also support the need for close contact. In a natural spawning event in calm water the sperm plume remained in a dense ribbon that ran over the animals, partly clinging to the shells and substratum until a horizontal surface was contacted causing it to slowly disperse. In a gentle swell, the sperm formed less coherent ribbons that spiralled in ever increasing dilute circles around and above the spawning individuals. In high flows in the aquarium, the released sperm were quickly moved horizontally in a narrow ribbon which dissipated within seconds. Of the few females observed spawning in the field, eggs exhibited similar behaviour to the sperm. In a gentle swell, the eggs appeared to be released with mucus and remained in a loose ribbon-like state but moved in increasing circles around and above the spawning individual, gradually becoming dispersed. In high flows, eggs dispersed rapidly in the water column. Aggregations of males and females for spawning purposes have also been reported in free-living deep sea invertebrates in order to increase sperm:egg encounters (Young et al. 1992).

Whilst high concentrations of sperm seem necessary for high fertilisation success in these Antarctic species, excessive concentrations can be detrimental to development. The presence of abnormally developing larvae for some mollusc species when sperm concentrations have exceeded 10^4 to 10^6 sperm ml^{-1} have been attributed to polyspermy (Gao et al. 1990, Mill & McCormick 1992, Clotteau & Dube 1993). In *Nacella concinna* the classical indication of polyspermy occurred with a sudden switch from normal to abnormal development at sperm concentrations $>10^6$ sperm ml^{-1}, followed by a fall in fertilisation success at the highest sperm concentrations. In contrast, *Laternula elliptica* showed only a small increase in abnormally developing embryos above 10^6 sperm ml^{-1}. The difference in proportions of abnormal to normal embryos recorded for *N. concinna* and *L. elliptica* could reflect the effectiveness of their specific polyspermic blocks, differences in their egg morphology or susceptibility to mechanical disturbance. Studies of the acrosome reaction and fertilisation in *Laternula limicola* have claimed that polyspermic penetration in this species is restrained by the creation of a perivitelline space in the egg investment (Hosokawa & Noda 1994). Thus, it may be possible that abnormalities caused by polyspermy are less frequent in the Laternulidae. The eggs of *L. elliptica* also possess a thick membrane compared to those of *N. concinna*, which could make them less susceptible to physical damage.

One explanation for the small increase in abnormalities and the strongly reduced levels of fertilisation success at the highest sperm concentrations in *Laternula elliptica* is oxygen depletion. Gruffydd & Beaumont (1970) reported large numbers of abnormally developing embryos and low yields at high sperm concentrations when using small culturing dishes, and concluded the effects were a result of oxygen deficiency and accumulation of waste products. Oliver & Babcock (1992) also suggested that decreased oxygen,

increased CO_2 and lower pH would cause a large decrease in fertilisation success. In the *L. elliptica* experiments oxygen levels decreased rapidly with increasing sperm concentration, although in 1 trial, no decrease in success or increase in abnormal development was observed even at the highest sperm concentration (10^8 sperm ml^{-1}). Thus, the sensitivity of gametes to environmental stress could vary between individuals. However, as high fertilisation success in *L. elliptica* was only achievable at sperm concentrations of ~10^7 sperm ml^{-1}, our data suggest that the eggs of *L. elliptica* not only require a very high spermatozoa to egg ratio to initiate fertilisation, but that they can also withstand a low oxygen environment during fertilisation.

Data here indicate that scoring abnormally developing embryos alongside normal development can reveal some of the underlying effects of sperm concentration and sperm ageing on fertilisation success, as well as highlighting problems with experimental design. It is common in fertilisation kinetics studies to score the presence of the fertilisation membrane as the indicator of fertilisation success before anomalies are apparent (Oliver & Babcock 1992, Benzie & Dixon 1994, Fong et al. 1995, Lasker et al. 1996, Encena et al. 1998, Babcock & Keesing 1999). However, fertilisation membranes can appear normal but produce embryos that develop abnormally during division (Farmanfarmaian & Giese 1963, cited in Greenwood & Bennett 1981). Using the presence of the fertilisation membrane alone may lead to an overestimation of the numbers of viable larvae surviving beyond early cleavage, and, hence, fertilisation success (Styan 1998b). Studies which score abnormal cleavages as unfertilised will clearly produce different estimates of fertilisation success from those using the presence of a fertilisation membrane.

In our study, total fertilisation success never reached 100% in any trial. Importantly, although values varied from 9 to 98%, a clear fertilisation pattern was still apparent. Variability in fertilisation rates from 0 to 100% is not uncommon in marine invertebrates and is a consequence of spawning in a dynamic environment and balancing sperm chemo-attraction whilst avoiding polyspermy (Levitan 1995).

Although abnormal development increased for *Nacella concinna* and *Laternula elliptica* above the optimum sperm density, indicating polyspermy and fertilisation inhibition, the costs of producing high quantities of sperm could be balanced by the ability to fertilise ova many days after release. Indeed, the spermatozoa from *L. elliptica* (at a concentration of 10^7 sperm ml^{-1}) were capable of fertilising fresh ova for more than 90 h after activation, and fertilisation rates of ~75% were still achievable after ~40 h. For *N. concinna*, fertilisation (at a concentration of 3.6×10^6 sperm ml^{-1}) was possible up to 65 h after release. By comparison, reported values of maximum sperm longevity in temperate molluscs are 5+ h for the abalone *Haliotis laevigata* at concentrations of 10^6 sperm ml^{-1} (Babcock & Keesing 1999), 2 h for *H. asinina* at 10^5 sperm ml^{-1} (Encena et al. 1998), 5+ h for the mussel *Mytilus edulis* (Levy & Couturier 1996) and 4 to 8 h in the cockle *Cerastoderma edule* (André & Lindegarth 1995). Sperm longevities of 48 and 24+ h respectively were reported in the ascidians *Ascidia mentula* (Havenhand 1991) and *Diplosoma listerianum* (Bishop 1998) and 20 h in the asteroid *Asterias rubens* (at 10^4 sperm ml^{-1}) (Williams & Bentley in press). Williams & Bentley also found increased sperm longevity in the polychaete, *Arenicola marina* at concentrations of 2.5×10^5 sperm ml^{-1} and 2.5×10^9 sperm ml^{-1} (80+ h), whilst the polychaete *Nereis virens* could fertilise fresh ova for ~24 and 120 h respectively at the same sperm concentrations. Based on work by Pacey et al. (1994) and Bishop (1998), Williams & Bentley (in press) hypothesised that high sperm concentrations (~10^9 sperm ml^{-1}) mimic conditions within the coelomic cavity of the polychaete thereby encouraging spermatozoa to remain quiescent, save energy and, thus, increase sperm longevity. Extremely long-lived sperm has been reported for deep sea echinoids, supposedly in response to low population densities and the lack of environmental cues (Levitan & Petersen 1995); however, this is not the case for increased sperm longevity in Antarctic clams and limpets.

Bolton & Havenhand (1996) reported decreased sperm longevity in temperate ascidians when holding the sperm in egg water. No such investigations were conducted here. However, as sperm often appears before eggs in the water column for our study species (D. Powell pers. obs., R. Luxmoore pers. comm.), the influence of egg water may not be as ecologically important. Conversely, we cannot rule out the effect of concentrating sperm in an experimental receptacle, which could reduce swimming activity, giving the impression of increased longevity. Our methodologies are, however, comparable with previous investigations, and our data do reveal differences in the sperm behaviour between these Antarctic species and temperate molluscs. Even if the increased sperm longevity of Antarctic and also deep sea spermatozoa is only a result of low seawater temperature (Davenport 1995), the benefits are not reduced. Long-lived sperm can increase the effectiveness of synchronised mass spawning events by allowing time for sperm densities to reach the high levels needed for fertilisation success. This will be important for *Laternula elliptica* which, as a population, can spawn en masse vast quantities of gametes over several days, and for *Nacella concinna*, which aggregate and synchronise spawning events

into a narrow time-frame over the brief spring bloom period. However, the effectiveness of these strategies will be markedly affected by the prevailing environmental conditions, including temperature and salinity (Powell unpubl. data), as well as water movement.

It seems clear that for *Laternula elliptica* and *Nacella concinna* a high degree of synchrony in spawning activities, a tendency to spawn in a low flow regime to minimise gamete dispersal coupled with close contact between mature individuals, high sperm concentrations and increased sperm longevity are utilised to enhance fertilisation success in the cold Antarctic waters. However, selection for enhanced fertilisation success will always be balanced against another life history trait and constrained by their physiology (Levitan 1995). Therefore, further work is required to understand the underlying mechanisms of fertilisation and to ascertain if the differences found here from temperate and tropical species in relation to sperm concentration and sperm longevity are specific to the study species or are an Antarctic wide phenomenon.

Acknowledgements. We are grateful for the assistance and advice of Jon Ward and Min Gordon at the British Antarctic Survey HQ, Cambridge, and all the personnel at Rothera Research Station for their support, particularly the SCUBA team: Steve Dunkerley (DO), Craig Barnes (DO), Alice Chapman, Si Brockington, Keiron Fraser, Jenny Beaumont, Ian McDonald, Martin Warnock, Dave Bailey and Katrin Linse. Alice Chapman and Jenny Beaumont also maintained the aquarium facilities at Rothera Research Station. Prof. Andrew Clarke, Maria Baker, Dr Phil Yund, Prof. John Pearse, Dr. Mark Williams and the anonymous reviewer are thanked for their constructive comments on the draft manuscripts. This project was funded by a NERC CASE postgraduate studentship to D.K.P. (GT4/97/272/MAS), which is gratefully acknowledged, and by the British Antarctic Survey.

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Editorial responsibility: Roger Hughes (Contributing Editor), Bangor, Wales, UK

Submitted: May 24, 2000; Accepted: November 16, 2000
Proofs received from author(s): April 9, 2001