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SHORT COMMUNICATION

The benefits of polyandry in the free-spawning polychaete *Galeolaria caespitosa*

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multiple mating;
sperm competition.

Abstract

In many species, females are thought to benefit from polyandry due to the reduced risks of fertilization by genetically incompatible sperm. However, few studies that have reported such benefits have directly attributed variation in female reproductive success to the interacting effects of males and females at fertilization. In this paper, we determine whether male × female interactions influence fertilization *in vitro* in the free-spawning, sessile polychaete *Galeolaria caespitosa*. Furthermore, we determined whether polyandry results in direct fertilization benefits for females by experimentally manipulating the number of males contributing towards staged spawning events. To test for male × female interaction effects we performed an initial experiment that crossed seven males with six females (in all 42 combinations), enabling us to assess fertilization rates for each specific male–female pairing and attribute variation in fertilization success to males, females and their interaction. This initial experiment revealed a strong interaction between males and females at fertilization, confirming that certain male–female combinations were more compatible than others. A second experiment tested the hypothesis that polyandry enhances female reproductive success by exposing each female's eggs to either a single male's sperm (monandry) or the sperm from three males simultaneously (polyandry). We performed this second experiment at two ecologically relevant sperm concentrations. This latter experiment revealed a strong fertilization benefit of polyandry, independent of the effects of sperm concentration (which were also significant). We suggest that these direct fertilization gains arising from polyandry will constitute an important source of selection on females to mate multiply in nature.

Introduction

In most mating systems females mate repeatedly with several males (polyandry), often exceeding the mating rate at which fertilization is assured (Birkhead & Møller, 1998). Because mating inevitably involves costs for both sexes, the adaptive basis of polyandry is hotly debated, especially where males contribute no material resources other than sperm at reproduction (Keller & Reeve, 1995; Yasui, 1997; Jennions & Petrie, 2000). In these resource-free mating systems, a number of hypotheses have been proposed to explain the occurrence and maintenance of polyandry. For example, under sperm limited conditions, natural selection may favour females that mate multiply if it allows them to maintain adequate sperm stores to ensure complete fertilization of current or subsequent egg clutches (Fjerdingstad & Boomsma, 1998; Kraus *et al.*, 2004). Other explanations for polyandry focus on how sperm competition (Parker, 1998) and cryptic female choice (Thornhill, 1983; Eberhard, 1996) can facilitate the receipt of indirect (genetic) benefits for females (Jennions & Petrie, 2000). For example, where males of high genetic quality produce competitively superior ejaculates (Hosken *et al.*, 2003), females may benefit from multiple mating through the enhanced genetic fitness of their progeny (Keller & Reeve, 1995; Yasui, 1997). Alternatively, multiple mating may enable

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females (or their eggs) to select compatible mating partners through male × female interactions at fertilization (e.g. Lewis & Austad, 1990; Wilson *et al.*, 1997; Clark *et al.*, 1999; Nilsson *et al.*, 2003).

Increasingly, studies reporting genetic benefits of polyandry indicate that females benefit from mating multiply by insuring against the risk of fertilization by genetically incompatible sperm (see reviews by Zeh & Zeh, 1996, 1997, 2003; Tregenza & Wedell, 2000). Studies from marine broadcast, or 'spermcast' species (*sensu* Pemberton *et al.*, 2003) and flowering plants provide some of the best examples of how sexual selection, mediated by gamete interactions, can select for genetically compatible mates. For example, in the colonial compound ascidian *Diplosoma listerianum*, females can discriminate amongst self- and nonself-sperm through phagocytosis in the oviduct (Bishop, 1996), thus blocking fertilization by self-sperm (Bishop *et al.*, 1996). Likewise, many angiosperms possess mechanisms that enable them to avoid self-pollination (reviewed by Barrett, 2003; Bernasconi *et al.*, 2004). In marine free-spawners, where both sexes shed gametes into the sea, compatibility-based selection through mutual gamete recognition is also well known (Vacquier & Moy, 1977; Whitaker & Swann, 1993; Vacquier, 1998). In sea urchins, the sperm protein bindin mediates gamete recognition and strongly influences fertilization success, both amongst closely related species (Metz *et al.*, 1994; Levitan, 2002; McCartney & Lessios, 2002) and within individual species (Palumbi, 1999). Importantly, the proteins that mediate fertilization in sea urchins and other free-spawners can exhibit substantial intraspecific variation (Metz & Palumbi, 1996; Biermann, 1998; Riginos & McDonald, 2003), therefore influencing gamete recognition at the individual level (e.g. Palumbi, 1999). Since assortative pairings between compatible mating partners is unlikely to occur through active mate discrimination in free-spawners, the sperm–egg interaction will represent the first opportunity for females to 'select' compatible mating partners (see Vacquier, 1998).

Given the importance of gamete compatibility in free-spawners, polyandry should benefit females because it is more likely to result in favourable associations between compatible eggs and sperm than monandrous pairings. Despite the inherent simplicity of this idea and its obvious relevance for free-spawners, the benefits of polyandry have rarely been examined in these and other externally fertilizing species (frogs: Byrne & Roberts, 2000; sea urchins: Evans & Marshall, in press). Here, we examine the consequences of polyandry for fertilization success in the free-spawning, sessile polychaete *Galeolaria caespitosa*. We first determined whether there was any indication of genetic incompatibility between different combinations of males and females. To do this, we used a simple diallel design, crossing the sperm and eggs of six females and seven males during staged fertilization trials, holding factors such sperm concentration and gamete contact times constant. This initial experiment revealed a strong interaction between male and female identity on fertilization success, indicating that certain male–female combinations were more compatible than others. We then tested the hypothesis that polyandry will result in enhanced fertilization success by exposing the eggs from individual females to either a single male's sperm (monandry) or the sperm from multiple males simultaneously (polyandry) at two ecologically relevant sperm concentrations.

Materials and methods

Natural history of *Galeolaria caespitosa*

Galeolaria caespitosa is a dioecious serpulid polychaete worm in which eggs and sperm are shed into the water column. *Galeolaria caespitosa* occurs in mixed sex clusters at a wide range of population densities on moderately exposed to high-energy shores in the intertidal zone of south-eastern Australia. As with most marine invertebrates, there are no data available on natural fertilization rates in the field. However, studies on other intertidal free-spawners have shown that fertilization success is highly variable amongst individuals, and that ambient sperm concentrations can vary from sperm limited conditions through to concentrations that cause polyspermy (Marshall, 2002; Marshall *et al.*, 2004). The eggs of *G. caespitosa*, like other intertidal polychaetes, are likely to experience a wide range of sperm concentrations with a similarly wide range in the number of males that contribute sperm to any particular spawning event (Williams *et al.*, 1997).

General methods

During April/May 2004, reproductively mature *G. caespitosa* adults were collected from pier pilings at Bare Island, Botany Bay, Australia. To collect gametes, individuals were removed from their calcareous tubes and placed in their own 40 mm Petri dish containing 3 mL of filtered (0.45 μm) seawater. Reproductively mature *G. caespitosa* begin spawning immediately after being removed from their tubes. We used a pipette to collect the spawned gametes and used them for experiments within 15 minutes of collection. Throughout all the experiments 0.45 μm filtered seawater was used and the fertilization assays were performed in clean, polyethylene vials using a total volume of 7 mL of the egg–sperm solution. All experiments were carried out under ambient temperature conditions ranging from 21 °C to 24 °C during the experimental period.

For all the experiments, eggs were exposed to sperm for 15 minutes. Following this, eggs were removed from the sperm solution, gently rinsed with filtered seawater on a 25 μm nitex mesh and placed in new vials. To assess fertilization success, we examined the eggs under a dissecting microscope 2 hours after they had initially been exposed to sperm. For each replicate vial, 100 eggs were examined; eggs were classed as fertilized if they had begun to undergo cell division and unfertilized if they had not begun to divide. During our experiments, we kept a subsample of eggs from each female in filtered seawater as a control for any errant fertilizations. When this control showed >1% fertilization success ($n = 1$ female), we discarded that batch of eggs. Sperm concentrations were estimated by doing three replicate counts of sperm for each sperm solution with a modified Fuch–Rosenthal haemocytometer.

Experiment 1: Effect of male and female identity on fertilization success

To test for male × female interactions at fertilization, we crossed the sperm and eggs from six females and seven males (in all 42 combinations) during artificial fertilizations. In this experiment, the eggs from each female were exposed to the sperm from each male using the same sperm concentrations and sperm–egg ratios. This was done by splitting individual egg clutches and sperm ejaculates and diluting gamete solutions from each individual to the appropriate concentration. For these fertilizations we used a sperm concentration of 4×10^5 sperm mL^{-1} and an egg concentration of 200 eggs mL^{-1}. For each male–female combination, we used two replicate vials to ensure that fertilization rates were repeatable for specific male × female crosses. Fertilization success was then assessed as described above.

Experiment 2: Effect of monandry/polyandry and sperm concentration on fertilization success

The first experiment revealed a strong interaction between males and females at fertilization (see Results), indicating that some male × female crosses were more successful than others. In a second experiment, we tested the prediction that because of these specific combining abilities between male and female gametes, eggs exposed to the sperm of multiple males (polyandry treatment) will exhibit higher fertilization rates than those exposed to sperm from single males (monandry treatment). Furthermore, we tested whether there was a benefit of polyandry at two sperm concentrations (nominally low and high – see below).

We used a paired design so that male–female identities were identical within each replicate under the monandrous and polyandrous treatments. For each replicate, the sperm of three males were used to fertilize the split egg clutch from a single female. The female's egg clutch was split into eight vials; two replicate vials were prepared for each male × female cross-under the monandry treatment, and a further two replicate vials were prepared for the combined ejaculates from each of the males in the polyandry treatment (see Fig. 1). This yielded a total of eight *in vitro* fertilizations for each three male × one female block. For six females we repeated this treatment at a high sperm concentration (1.5×10^6 sperm mL^{-1}) and for six females, a low sperm concentration (7×10^4 sperm mL^{-1}) yielding a total of 192 fertilizations. Note that a different male sperm source was used for each replicate, resulting in 36 (12 female blocks × three males) different males being used.

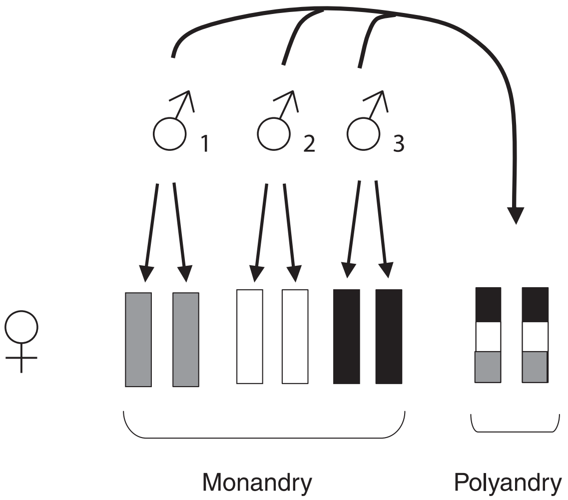

Fig. 1 Schematic overview of the methods used for experiment 2. The eggs of each female were exposed to the two replicate vials of sperm from each male (monandry) and two replicate vials of a mixed sperm solution from all three males (polyandry). The sperm solutions were constant across each male and treatment. This experiment was repeated using the same males and females at two sperm concentrations (see text for details).

Across both the polyandry/monandry and sperm concentration treatments, eggs were adjusted to the same concentration (1000 eggs mL^{-1}). In the monandry treatment, the concentrations of sperm from different males were identical within each sperm concentration treatment. In all cases sperm solutions were made up to a final volume of 6 mL. In the polyandry treatment, 2 mL of sperm solution from each male was combined to produce a mixed sperm solution (final volume = 6 mL) in each vial (yielding an identical volume and concentration to the monandry vials). The treatments were then applied as described in Fig. 1: for each female, there were six monandry vials (two replicate vials for each male) and two polyandry vials (two replicate vials of the mixed sperm solution). Average fertilization success of each female under monandry (from the six replicate vials) and polyandrous conditions (from the two replicate vials) at both sperm concentrations was then compared (see below).

Statistical analysis

In experiment 1, the effect of male and female identity (and their interaction) on fertilization success was examined using a two-way ANOVA where both male and female identity were random factors. In experiment 2, we compared fertilization success under monandry and polyandry at the two sperm concentrations with a partly-nested ANOVA (Quinn & Keough, 2002). Sperm concentration and monandry/polyandry were both fixed factors (two levels in each) and female identity was a random factor nested within sperm concentration. We could have analysed arcsine square-root transformed data but the data were not badly skewed and transformation affected neither the distribution of the data nor the outcome of the analyses.

Results

Experiment 1

When sperm concentrations were kept constant, fertilization success in *G. caespitosa* was strongly dependent on the interaction between males and females (Table 1). Certain male × female crosses exhibited higher fertilization successes than others, despite all other conditions being the same. For example, the fertilization rate of female 6's eggs using male 6's sperm was $\sim$35%. However, this low value was not because of either poor sperm or unviable eggs because female 6's eggs achieved $\sim$80% fertilization with male 4's sperm and male 6's sperm fertilized $\sim$85% of female 5's eggs (Fig. 2). These effects were repeatable across replicates as indicated by the significant interaction term in Table 1.

Table 1 Two-way ANOVA on the effect of male and female identity on the fertilization success of *Galeolaria caespitosa* eggs.

Source	D.f.	MS	*F*	*P*-value
Female	5	0.178	5.24	0.001
Male	6	1.099	32.31	<0.001
Male × female	30	0.034	5.875	<0.001
Residual	42	0.006		

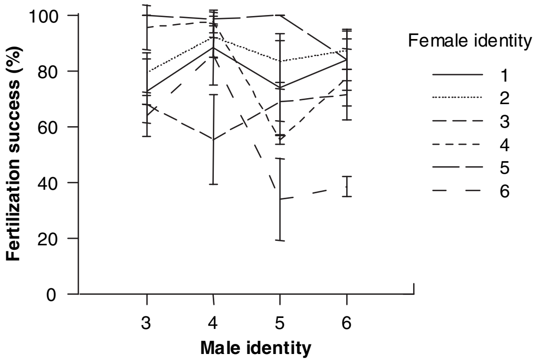

Fig. 2 The interaction between male and female identity at fertilization in *Galeolaria caespitosa*. Each point represents the mean (±SE) fertilization success of eggs for two replicate vials per female. For clarity, the results of crosses from four males only are shown.

Experiment 2

At both sperm concentrations, eggs were fertilized at a significantly higher rate under the polyandry treatment compared with the monandry treatment (Table 2). Our analysis also revealed no significant interaction between sperm concentration and the polyandry/monandry treatments, indicating that the benefits of polyandry are similar at both low and high sperm concentrations. On average, the fertilization rate of eggs under polyandry was 10% higher than that of the same eggs with the same males under monandry (Fig. 3).

Table 2 The results from a partly nested ANOVA testing the effect of sperm concentration (high = 1.5×10^6 sperm mL^{-1}; low = 7×10^4 sperm mL^{-1}) and polyandry/monandry treatment and their interaction on fertilization success in *Galeolaria caespitosa*.

Source	D.f.	MS	*F*	*P*-value
Between subjects				
Sperm concentration	1	22858	164.936	<**0.001**
Error	10	138		
Within subjects				
Treatment	1	256	9.76	**0.011**
Treatment × sperm concentration	1	0.026	0.001	0.975
Error	10	26.42		

Significant *P*-values are highlighted in bold.

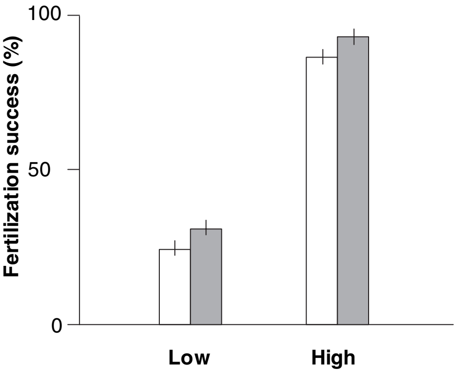

Fig. 3 The effect of polyandry and sperm concentration on the fertilization success of *Galeolaria caespitosa* eggs. Each bar represents the mean fertilization success of females under polyandry (indicated by shaded bars) and monandry (indicated by open bars). Bars on the left represent trials conducted using a low sperm concentration and bars on the right represent trials using a high sperm concentration (see text for details). The error bars represent the square root of the mean square error from the partly-nested ANOVA in Table 2.

Discussion

Our finding in the first experiment that variation in fertilization success in *G. caespitosa* can be explained by the interacting effects of male and female compatibility compliments preliminary work done on this species (Kupriyanova & Havenhand, 2002) and adds to an increasing body of evidence that sperm utilization by females is nonrandom with respect to male identity (e.g. Clark *et al.*, 1999; Palumbi, 1999; Miller & Pitnick, 2002; Nilsson *et al.*, 2003). An important consequence of these male × female interactions was revealed by our second experiment: females that spawn in the presence of several males will increase the likelihood that their eggs will be fertilized by the most compatible males and will therefore benefit directly from polyandry. Our results also revealed a strong effect of sperm concentration on fertilization success, although the magnitude of the fertilization benefit derived from polyandry did not depend on the sperm concentrations used in the two treatments (as evident by the noninteracting effects of sperm concentrations and polyandry treatment; Table 2). These direct fertilization gains arising from polyandry may constitute an important source of selection on females to mate multiply in nature.

Spawning aggregations (both temporal and spatial) are traditionally viewed as the product of naturally selected behaviours that increase the reproductive success of both sexes (Brawley, 1992; Brawley *et al.*, 1999; Yund, 2000). Indeed, many authors emphasize the importance of population density, spawning synchrony and the production by males of high numbers of sperm in reducing the negative effects of sperm limitation (reviewed by Yund, 2000). Although our results support this view by showing that sperm concentration has a strong influence on fertilization rates (in both treatment groups), they also indicate that when this and other factors are held constant, polyandry (i.e. the number of males contributing to a spawn) will also significantly influence on fertilization rates, even at apparently low sperm concentrations. Genetic incompatibility may explain why sometimes female fertilization success can be low even when spawning males are relatively nearby and sperm are abundant (e.g. see Fig. 2 in Marshall *et al.*, 2004).

An important challenge for future work on *G. caespitosa* is to identify the mechanisms that account for specific interactions between males and females at fertilization. In other free-spawning marine invertebrates a number of studies have emphasized the role of sperm surface proteins as cues for sperm–egg recognition (reviews by Vacquier, 1998; Swanson & Vacquier, 2002; Bernasconi *et al.*, 2004). For example, in sea urchins positive selection for loci that code for polymorphic sperm proteins (bindin) (Metz & Palumbi, 1996) facilitates species–specific gamete interactions (Metz *et al.*, 1994) and may fuel reproductive isolation amongst closely related congeners (Palumbi & Metz, 1991). Importantly, these gamete recognition proteins can also be highly variable within species (Metz & Palumbi, 1996; Biermann, 1998; Riginos & McDonald, 2003), resulting in positive assortative fertilizations between eggs and sperm with similar genotypes (Palumbi, 1999).

Amongst sedentary free-spawners, where gamete interactions are likely to be the only form of mate choice available to females, variation amongst individuals in gamete recognition proteins may have important implications for the evolution of spawning behaviour. Our current findings for *G. caespitosa*, in conjunction with our recent experimental work on the Australian sea urchin *Heliocidaris erythrogramma* (Evans & Marshall, in press), illustrate this point by showing that when sperm concentration and sperm–egg contact times are held constant, polyandry increases fertilization rates dramatically. Taken together, these studies suggest that in addition to the strong naturally-selected advantages of group spawning (e.g. avoidance of sperm limitation; Brawley, 1992; Brawley *et al.*, 1999; Yund, 2000), polyandry will confer important sexually-selected advantages on females via the ability to select compatible mating partners at fertilization (e.g. Zeh & Zeh, 1996, 1997). Our results additionally suggest that the magnitude of the fertilization benefit derived from multiple mating will positively covary with the extent of intraspecific variation in sperm–egg recognition molecules. Hence, the reproductive benefits of polyandry, at least with respect to fertilization, should be greatest in species (or populations) in which positive selection increases intraspecific variability in gamete cell recognition molecules (as for example in abalone: Galindo *et al.*, 2003; teguline gastropods: Hellberg *et al.*, 2000; and see urchins: Metz & Palumbi, 1996; Zigler *et al.*, 2003). We eagerly await population- and species-level comparisons of the benefits of polyandry to test these predictions explicitly.

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