

The early sperm gets the good egg: mating order effects in free spawners

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Mating order can have important consequences for the fertilization success of males whose ejaculates compete to fertilize a clutch of eggs. Despite an excellent body of literature on mating-order effects in many animals, they have rarely been considered in marine free-spawning invertebrates, where both sexes release gametes into the water column. In this study, we show that in such organisms, mating order can have profound repercussions for male reproductive success. Using *in vitro* fertilization for two species of sea urchin, we found that the 'fertilization history' of a clutch of eggs strongly influenced the size distribution of unfertilized eggs, and consequently the likelihood that they will be fertilized. Males that had first access to a batch of eggs enjoyed elevated fertilization success because they had privileged access to the largest and therefore most readily fertilizable eggs within a clutch. By contrast, when a male's sperm were exposed to a batch of unfertilized eggs left over from a previous mating event, fertilization rates were reduced, owing to smaller eggs remaining in egg clutches previously exposed to sperm. Because of this size-dependent fertilization, the fertilization history of eggs also strongly influenced the size distribution of offspring, with first-spawning males producing larger, and therefore fitter, offspring. These findings suggest that when there is variation in egg size, mating order will influence not only the quantity but also the quality of offspring sired by competing males.

Keywords: sexual selection; offspring size; sperm precedence

1. INTRODUCTION

Numerous studies have revealed an effect of mating order on the outcome of sperm competition, which occurs when the ejaculates of two or more males compete for the fertilization of a female's eggs (Parker 1970). Although documented for a wide range of taxonomic groups (Smith 1984; Birkhead & Møller 1998; Simmons 2001), including sessile marine invertebrates (Bishop *et al.* 2000), the influence of mating order on sperm use has never been described in free-spawning marine invertebrates, where both sexes shed gametes into the water column. Indeed, in free-spawning marine invertebrates, sexually selected forces are often considered of secondary importance to naturally selected processes such as sperm limitation (reviewed in Yund 2000). Nevertheless, there is clearly the potential for sperm competition, mating-order effects and sperm precedence in free-spawning animals (Gage *et al.* 2004). For example, during natural matings in sea urchins, spawning females are typically surrounded by multiple males and eggs can remain *in situ* for prolonged periods accumulating fertilizations from a number of males (e.g. Yund & Meidel 2003). Furthermore, there is a clear but untested mechanism for mating-order effects in free spawners. For example, when eggs are spawned into the water column, their probability of encountering sperm is influenced strongly by their size; larger eggs are more likely to be contacted by sperm than smaller eggs (Levitan 1996; Styan 1998; Marshall *et al.* 2002). Consequently, the largest, most fertilizable eggs (i.e. fertilized at low sperm concentrations) produced by a female are likely to be encountered by sperm that arrives first, leaving only

the smaller less fertilizable eggs for subsequent sperm to encounter. Despite the implications of egg-size variation for fertilization success, the idea that males should enjoy enhanced fertilization gains by ensuring mating primacy has not, to our knowledge, been examined in any free-spawning marine invertebrate. Moreover, the potential for mating-order effects in such species is of interest because of the insights it could provide into the role of sexual selection in the evolution of internal fertilization from the ancestral mating strategy of free spawning.

Here, we investigate the effects of mating order on direct components of male reproductive success using *in vitro* fertilization techniques for two species of sea urchin, *Heliocidaris erythrogramma* and *Holopneustes purpurescens*. Specifically, we examined the consequences of mating order for fertilization success and offspring size. We selected these species because sea urchins, with their ancestral reproductive strategy, are model organisms for the study of fertilization (Levitan 1996; Zigler *et al.* 2003). Moreover, sea urchins are particularly amenable to this investigation because egg size is highly variable within species (Lessios 1987), and strong effects of egg size on post-metamorphic fitness have been demonstrated for a number of species, including *He. erythrogramma* (Emlet & Hoegh-Guldberg 1997; Marshall *et al.* 2003). Both species examined here have non-feeding larvae (a planktonic period of 4–15 days), and therefore egg and larval size reflects the total amount of maternal investment in each young.

2. METHODS

(a) *Experimental overview*

The aim of this experiment was to determine if the fertilization success of individual males was affected by the previous 'fertilization history' of a clutch of eggs. To do this we used a

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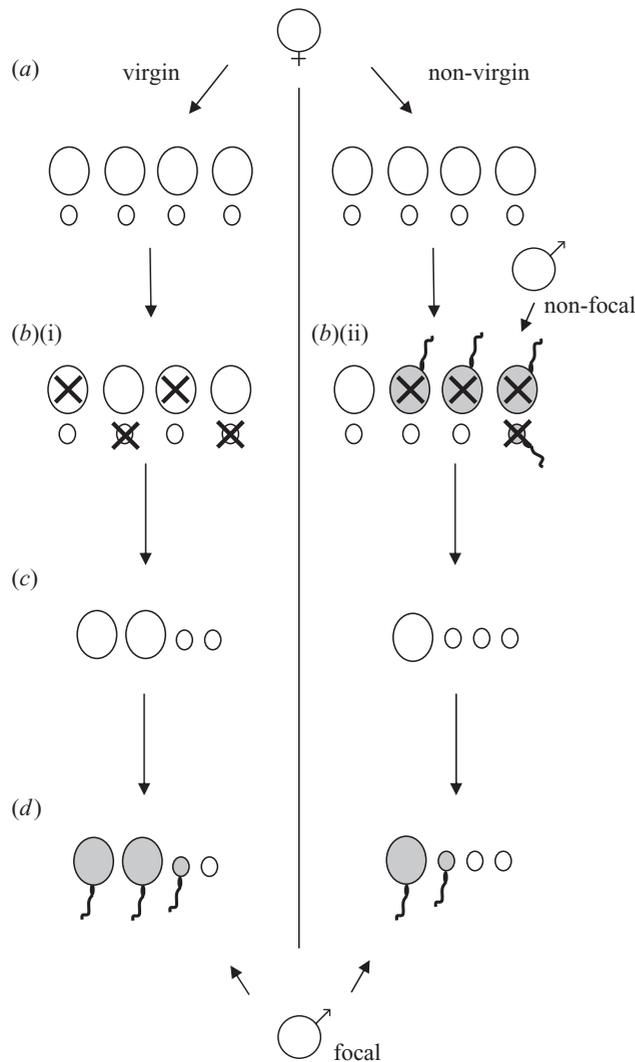


Figure 1. Schematic overview of the experimental methods and predictions for the effect of fertilization history on male reproductive success. (a) The eggs (large and small circles) of a female are split into two groups, virgin and non-virgin. (b)(i) A random sample of the virgin eggs is removed and placed into a new container, and (ii) a non-virgin group of eggs is exposed to the sperm of a non-focal male; within this group, the larger eggs are more likely to be fertilized (indicated by shading) than the smaller eggs. The remaining unfertilized, smaller eggs are then removed and placed into a new container. (c) In the new container, both groups of eggs are measured and the number of eggs in each group is adjusted to be the same in both groups. The mean size of the virgin eggs is predicted to be greater than that of non-virgin eggs. (d) Both groups are exposed to the ejaculates from the same focal male. Because of the size difference between the groups of eggs, the ejaculate exposed to the virgin eggs is predicted to fertilize more eggs and produce larger offspring than the ejaculate exposed to the non-virgin eggs. Circles represent eggs, black squiggles represent sperm.

split-clutch and split ejaculate design (figure 1). Specifically, eggs from the same clutch were allocated, in equal numbers, to either a 'virgin' or a 'non-virgin' group. We use the term 'virgin' to describe eggs that had not been exposed previously to sperm, and 'non-virgin' for eggs that had previously been exposed to sperm but remained unfertilized. We split the ejaculate of individual males (referred to as focal males) and compared the relative fertilization success of the ejaculate portions with a split

clutch of eggs from an individual female. One half of the clutch was incubated in filtered seawater (hereafter termed the 'virgin' eggs) before being exposed to the focal male's sperm. The other half of the clutch of eggs was incubated in seawater containing the sperm of another male (referred to as the non-focal male). The eggs that were not fertilized during this incubation with the non-focal male (hereafter termed 'non-virgin' eggs) were then isolated and exposed (at the same concentration as for the virgin eggs) to the sperm from the focal male. Thus, both portions of the ejaculate from the focal male had access to the same concentration of unfertilized eggs and this produced two fertilization estimates for each male: success with virgin eggs and success with non-virgin (but unfertilized) eggs. We also measured the effect of an egg clutch's fertilization history (i.e. virgin versus non-virgin) on (i) the size distribution of unfertilized eggs available to the split ejaculates of individual focal males and (ii) the size distribution of offspring sired by males whose ejaculates had access to either virgin or non-virgin eggs. Our general methods and predictions are depicted in figure 1. The above general protocol describes the methods used for each 'block' of one female and two males (i.e. the split clutch of one female's eggs and the ejaculates from each of two males: focal and non-focal). For the fertilization measures we performed 10 blocks (i.e. replicates) for each species, whereas for the offspring size measures we performed five blocks for each species.

(b) Gamete collection

Reproductively mature adults of both species were collected from Bare Island, Sydney, Australia (151°23' E, 33°99' S) during November and December 2003. Upon collection, *He. erythrogramma* were induced to spawn with a 5 ml injection of 0.5 M KCl. The gametes of *Ho. purpurascens* were collected by dissecting out the gonad and gently tearing it into small pieces. Sperm was collected 'dry' (i.e. no seawater was added to the ejaculate), and the positively buoyant eggs were collected after immersing the dissected gonads in 10 ml of 0.2 µm-filtered seawater. The eggs were rinsed in 0.2 µm-filtered seawater and left for 30 min to allow the expansion of their jelly coats (expanded jelly coats on the outside of eggs have been shown to enhance fertilization (Podolsky 2001)).

(c) Manipulating the fertilization history of eggs

Depending on the treatment, all experiments were carried out in 25 ml of solution that contained either filtered (through 0.2 µm pore size) seawater or sperm mixed with filtered seawater. We created an 'intermediate' sperm concentration (one that results in *ca.* 50% fertilization success) throughout our experiments. There are no available data on natural sperm concentrations for either species so we *a priori* selected a concentration that would not result in all the eggs in the batch being unfertilized (i.e. a very low sperm concentration) or fertilized (i.e. a very high sperm concentration). The fertilization rates used in this study are typical of marine free spawners (Marshall 2002; Marshall *et al.* 2004). Following collection, the eggs ($n \approx 2000$) of each female were split into both a non-virgin group, where eggs were exposed to a 2×10^3 sperm ml⁻¹ solution (that was less than 15 min old) from the non-focal male for 15 min, and a virgin group, where eggs were exposed to the same volume of filtered seawater. After 15 min, *ca.* 400 unfertilized eggs from each group were removed and placed in new Petri dishes (for the group that had been exposed to sperm this meant collecting individual unfertilized eggs under a dissecting

microscope). The eggs were classified as unfertilized if no fertilization membrane was apparent, which proved to be a reliable indicator of fertilization (see below). At this point there were two groups of unfertilized eggs: those eggs that had been exposed to the sperm of the non-focal male but had not been fertilized (the non-virgin group) and those that had not been exposed to any sperm but had been treated the same in every other way (the virgin group). A small ($n = 20$), haphazardly selected subsample of eggs from each group was then measured (diameter to the nearest $4 \mu\text{m}$) using a dissecting microscope attached to a computer. This produced an estimate of the size of eggs available in each group for fertilization by the split ejaculates of the focal male.

(d) Controls

Controls were performed for each block to ensure that the eggs that were classified as unfertilized in the non-virgin group were viable (i.e. capable of being fertilized) and were indeed unfertilized. To confirm that eggs were viable, a subsample (100 eggs) was repeatedly exposed to an intermediate sperm concentration (2×10^3 sperm ml^{-1}) from the focal male. This repeated exposure of eggs to newly activated sperm maximizes the chances of fertilization (Marshall 2002). Fertilization success was assessed after 1 h. The average positive control fertilization rate was very high (pooling for both species: mean 95.4%; s.e. 0.44%; range 92–99%; $n = 20$), indicating that the non-virgin eggs available to the focal male were viable. A subsample of 100 eggs was removed from the non-virgin group, placed in filtered seawater and monitored for the next 2 h for any sign of fertilization or development (there was no fertilization in any replicate).

(e) The assessment of fertilization success and offspring size

Approximately 200 eggs from both treatment groups (virgin and non-virgin) were each exposed to a 2×10^3 sperm ml^{-1} sperm solution from the focal male for 15 min. For both treatment groups, the age, concentration and source of sperm used for the fertilizations were identical. The percentage of eggs fertilized was then assessed for both groups. For both species, fertilized eggs from five male–female blocks were placed into fresh seawater cultures (5 ml of filtered seawater at a constant temperature of 20°C) and allowed to develop for 48 h. For the examination of offspring size, a subsample of the resultant larvae ($n = 20$) from each group (virgin and non-virgin) was measured (individual larval length to the nearest $4 \mu\text{m}$). We examined the larvae of only five male–female blocks because we had previously observed strong effects of our treatments on the size distribution of eggs available to the focal male (see § 3).

3. RESULTS

Males that were able to fertilize virgin eggs first had significantly higher fertilization success than those that had access to unfertilized eggs that were left over from a previous mating event (paired t -tests: *Heliocidaris*: $t = 3.60$, $p = 0.006$, d.f. = 9; *Holopneustes*: $t = 4.68$, $p = 0.001$, d.f. = 9; figure 2). The size of eggs that were available to males that had first access to eggs was significantly larger than that of those that were left over and available to the second male (*Heliocidaris*: $t = 7.11$, $p < 0.0001$, d.f. = 9; *Holopneustes*: $t = 7.75$, $p < 0.0001$, d.f. = 9). This size-dependent fertilization resulted in differences in the size of offspring sired by first and second males. The larvae

arising from eggs sired by males who spawned first were *ca.* 20% larger (volume difference calculated assuming larvae were elliptical) than their siblings produced from eggs sired by the second male for both species (*Heliocidaris*: $t = 2.94$, $p = 0.042$, d.f. = 4; *Holopneustes*: $t = 2.86$, $p = 0.046$, d.f. = 4; figure 2).

4. DISCUSSION

Our results clearly indicate that in free-spawning marine invertebrates the ‘fertilization history’ of a clutch of eggs can influence both the quantity and quality of offspring sired by males. Males whose sperm had access to ‘virgin’ eggs (i.e. previously unexposed to sperm from the non-focal male) had higher fertilization success than those whose sperm were exposed to unfertilized eggs left over from a previous mating event. These effects are a direct product of the kinetics of fertilization in free spawners and the fact that egg size is highly variable within clutches of eggs (Styan 1998; Marshall *et al.* 2002). The larger eggs were preferentially fertilized during the initial artificial mating event (with the non-focal male), leaving smaller eggs available for subsequent fertilizations by the focal male’s sperm. Smaller eggs are less likely to be contacted by sperm, and thus the overall fertilization rate will be lower than for virgin eggs exposed to the same sperm concentration. This was evident from the observation that in both species, the size of eggs available for fertilization after a staged mating event was smaller than that for a clutch of eggs previously unexposed to sperm. Inevitably, the spawning conditions imposed during our staged laboratory matings would have been more simplistic than those in nature. For example, in our experimental protocol, eggs that were fertilized by the first male were removed, leaving only fertilizable eggs for the sperm from subsequent males to encounter. During natural spawnings, however, previously fertilized eggs will remain, thus further reducing the effective concentration of sperm from subsequent males. Therefore, under natural conditions the effects of mating order on the fertilization success of the second male are likely to be greater than those observed here.

A second consequence of this size-dependent fertilization of eggs is that males who mate with females first will sire larger offspring. Previous work has revealed that even small differences in offspring size can have important post-metamorphic consequences in a range of marine invertebrate species, including *He. erythrogramma* (Emler & Hoegh-Guldberg 1997; Moran & Emler 2001; Marshall *et al.* 2003). Given that within-individual variation in egg size is common (Lessios 1987; Robertson 1996), mating-order effects on offspring quality may also be common among free spawners. The effects of mating order on male reproductive success and offspring fitness have important and previously unforeseen consequences. Males that optimize the timing of fertilization so that their sperm are first to interact with a female’s eggs will enjoy higher (direct) fitness returns because they will sire both more and better offspring. We suggest that this will result in strong directional selection on male traits (both behavioural and physiological) that optimize the timing of fertilization (e.g. Wedell & Cook 1998; Birkhead *et al.* 1999; LaMunyon & Ward 1999; Evans & Magurran 2001). Males that have the greatest competitive ability with

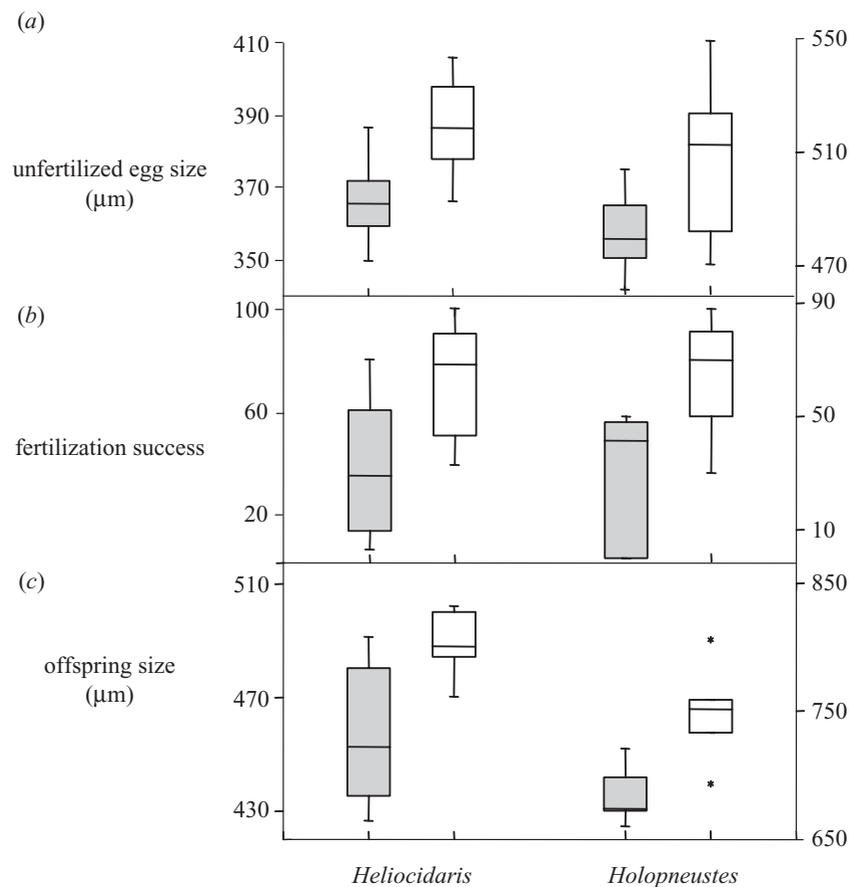


Figure 2. Effect of mating primacy on male reproductive success in two broadcast spawning sea urchins, *Heliocidaris erythrogramma* and *Holopneustes purpurascens*. Mating primacy influences: (a) mean size of eggs available for fertilization; (b) mean fertilization success (percentage of eggs fertilized in each batch); and (c) mean size of offspring. In each case, eggs with a virgin fertilization history are represented by open bars and eggs that were pre-exposed to another male are represented by shaded bars.

respect to mating primacy will secure the greater and highest-quality portion of a female's investment. Whether this disproportional maternal investment in favour of the most competitive males represents a form of female choice is unknown, but the potential for this choice to exist at all in free spawners was unanticipated.

To the extent that the production of large eggs by females is heritable (Azevedo *et al.* 1997), males that achieve mating primacy will also benefit indirectly by siring daughters that produce relatively large eggs (and consequently fitter offspring (Wolf *et al.* 1998)). Likewise, if male traits that influence fertilization order are heritable, females will benefit because their largest ova will be preferentially fertilized by sperm from the fittest males. Hence, the non-random association between 'good sperm' and 'good eggs' will have important consequences for sexual selection in broadcast spawning organisms and may favour the production by females of eggs that are larger than their naturally selected optima.

It has been well established that spawning too soon will result in sperm diluting to ineffective concentrations, and spawning too late will result in no offspring at all (Levitan 1995). However, our results suggest that in addition to these natural selection pressures for males to optimize the timing of spawning, there will also be strong sexual selection pressure on males to achieve mating primacy. This is likely to have important implications for the reproductive

ecology and evolution of free-spawners. Previously, aggregations of spawning individuals (e.g. Harrison *et al.* 1984) have been viewed purely as a product of natural selection to maximize fertilization success in both sexes (reviewed by Yund 2000). Our results suggest that sexual selection will also favour males who optimize the timing and position of sperm release. Male marine invertebrates tend to spawn before females and sometimes in the absence of any apparent cue (Levitan 1988; Strathmann 1991). This apparently maladaptive behaviour may be a response by males to pre-empt potential rivals in the competition for females. The transition from the ancestral mating strategy of free spawning to internal fertilization has traditionally been viewed as a product of natural selection (Levitan 1996). Given the extreme selection pressure for males to ensure that their sperm fertilize a clutch of eggs first (by getting as close to a spawning female as possible), it may be that sexual selection played a previously unforeseen role in the evolution of brooding and ultimately internal fertilization.

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