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ORIGINAL PAPER

Eggs with larger accessory structures are more likely to be fertilized in both low and high sperm concentrations in *Styela plicata* (Ascidiaceae)

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Abstract The evolution of egg size has been intensively studied due to its influence on both fecundity and offspring performance. In marine broadcast spawners, egg size influences the probability of sperm–egg collision, and therefore, egg size can also influence fertilization success, depending on the local concentration of sperm. Many broadcast-spawning species have egg accessory structures that are thought to be a cheap means of altering egg size, but their influence on fertilization remains controversial. To determine the relative influences of ovicell size and follicle cell size on fertilization success in the ascidian *Styela plicata*, the size distribution of eggs that were not successfully fertilized in both high and low sperm concentrations was compared to that of unfertilized controls. At high sperm concentrations, a greater proportion of eggs with smaller ovicells were fertilized, resulting in smaller larvae hatching from this treatment. Eggs with a large follicle cell area relative to ovicell area were preferentially fertilized in both high and low sperm concentration treatments. Hence, follicle cells do not eliminate selection on ovicell size at fertilization in *S. plicata*. Furthermore, follicle cells appear to increase fertilization success across a range of sperm concentrations by performing different functions in each environment—increasing the target size of eggs in low-sperm concentrations and presumably reducing polyspermy in high sperm concentrations.

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Introduction

Offspring size is a trait of fundamental evolutionary importance, as it has pervasive consequences for both offspring quantity and quality (Bernardo 1996). Mothers may invest in either many small eggs, or fewer large eggs (Smith and Fretwell 1974; Lloyd 1987). Larger offspring generally have greater fitness than smaller offspring, although the precise relationship between offspring size and performance depends on the local environment (Parker and Begon 1986; Lloyd 1987; Marshall et al. 2006; Allen et al. 2008; Allen and Marshall 2014).

The challenge of optimally provisioning eggs is compounded for organisms with complex life cycles, as each life-history stage can have different (potentially conflicting) optimal phenotypes (Moran 1994; Gagliano et al. 2007; Crean et al. 2011). In marine broadcast spawners (where both eggs and sperm are released into the water), in addition to effects on post-zygotic performance, egg size influences fertilization success (reviewed in Levitan 2006; Marshall and Keough 2008). The physical size of eggs influences the probability of sperm–egg collision (Levitan 1996). Because larger eggs are larger 'targets' for sperm, larger eggs are more likely to come into contact with a sperm and be fertilized in sperm-limiting conditions (Levitan 1993, 1996, 2004). However, at high sperm concentrations, larger eggs are more likely to come into contact with

multiple sperm and become polyspermic (a lethal condition that causes fertilization failure) (Styan 1998; Marshall et al. 2002; Levitan 2004). Thus, females are expected to adapt their egg size according to the predicted fertilization environment (Crean and Marshall 2008; Burgess and Marshall 2014).

In many marine invertebrates, egg accessory structures—such as jelly coats in echinoderms and molluscs and follicle cells in ascidians—surround the main ovicell, which provides all of the maternally derived nutrition requirements of the embryo prior to hatching (Podolsky 2004; Levitan 2006). Accessory structures are thought to be relatively cheaper to produce than ovicells (Bolton et al. 2000) and may therefore provide an energetically inexpensive means of increasing the egg target size, without large increases in overall investment per offspring (Bolton et al. 2000; Podolsky 2001; Randerson and Hurst 2001). Furthermore, as accessory structures may decouple the relationship between the physical and energetic egg size (Podolsky 2004), they may allow for selection to act on these two traits independently. However, in addition to increasing the physical egg size, accessory structures may serve several different functions such as altering sperm behaviour, protection from shear forces, floatation, prevention of self-fertilization, and/or promoting species specificity (Villa and Patricolo 1993; Levitan 2006; Lambert 2009). The role of accessory structures and how they influence egg-size-dependent fertilization success across a range of sperm environments remain unclear.

If the main function of accessory structures is to increase the physical size of eggs, they should be selected for when fertilization is limited by low-sperm concentrations (Farley and Levitan 2001; Podolsky 2004). However, whether accessory structures can act to buffer selection on ovicell size at fertilization remains a source of intense debate. Optimization models differ in their predictions, with one set of models predicting that egg accessory structures buffer ovicell size from selection driven by changes in the sperm environment (Podolsky 2004), while another suite of models predicts that the sperm environment will impose selection on ovicell size, regardless of the presence of accessory structures (Levitan 2000; Farley and Levitan 2001; Levitan 2006). Therefore, it remains unclear whether an increase in the area of accessory structures, independently of an increase in ovicell size, would increase fertilization success in sperm-limiting conditions.

Accessory structures may play a similarly important role in fertilization under conditions where sperm are in excess, but this possibility has received very little attention. It has been suggested that accessory structures increase the variance in sperm arrival times, thereby reducing polyspermy in high sperm concentrations (Podolsky 2001). In this case, eggs with a large area of accessory structures would be selected for in both high and low sperm concentrations. However, if accessory structures act solely to increase the probability of sperm–egg contact, then eggs with a smaller area of accessory structures will be selected for in polyspermy-inducing conditions.

Previously, we demonstrated that in *Styela plicata* (Ascidiaceae) both ovicell and follicle cell area of the eggs vary in relation to the local density of conspecifics (Crean and Marshall 2008). To demonstrate how these differences in egg traits are likely to influence fertilization success, we ran in vitro fertilization trials under high and low sperm concentrations and examined the properties of eggs that showed no sign of development. This indirect measure of fertilization success was used because fertilized eggs change shape after cleaving, and therefore, the size distribution of fertilized eggs could not be measured after development had commenced (Marshall et al. 2002). Ascidian eggs that suffer polyspermy typically fail to cleave (Lambert and Lambert 1981), and therefore, we were unable to distinguish between unfertilized and polyspermic eggs. However, as neither unfertilized nor polyspermic eggs will develop, the indirect measure used gives an estimate of the size distribution of eggs that will develop into larvae.

Materials and methods

Study species

Styela plicata is a broadcast-spawning, hermaphroditic (although largely non-self fertile), solitary ascidian. *Styela plicata* eggs consist of the main ovicell (size range of ovicell area = 14–28 × 10^3 μm^2), surrounded by large, columnar follicle cells (size range of follicle cell area = 11–26 × 10^3 μm^2) (Villa and Patricolo 2000; Crean and Marshall 2008). Fertilization can be replicated in the laboratory using established, standardized protocols (Crean and Marshall 2008; Crean et al. 2012). Fertilized eggs hatch into lecithotrophic (non-feeding) larvae within 10–12 h, which are competent to settle within a few hours (Yamaguchi 1975; Crean et al. 2011, 2013). Adult *S. plicata* used in this experiment were collected in March 2008 from Manly Boat Harbour (Brisbane, Australia; 27.467 E 153.183 S) and maintained in aerated seawater until use (within 2 d).

Gamete collection

Gametes were harvested using standard 'strip-spawning' techniques which do not appear to affect the integrity of accessory structures in this species (Marshall et al. 2000; Crean and Marshall 2008). Briefly, the gonads were dissected from each individual into a Petri dish with a few

drops of filtered seawater (FSW). This gonad extract was gently pressed with a rubber stopper to release the gametes and poured through a 100-μm filter with FSW into a small beaker. This procedure allowed the eggs to be retained inside the filter, while the sperm passed through into the beaker. Individuals were randomly designated as either 'male' (only sperm collected) or 'female' (only eggs collected).

Effects of egg size on fertilization success in high and low sperm concentration assays

Ten paired in vitro fertilization trials were run in seawater collected from the field to compare the size distribution of eggs that were successfully fertilized in high and low sperm concentrations. In each trial, 6 mL of eggs were harvested from a female and split evenly among three Petri dishes. Five millilitres of sperm was then collected from each of two males (to reduce genetic and compatibility effects) and mixed together to form the high sperm concentration treatment solution (mean sperm concentration = 7.2×10^7 sperm mL^{-1}, range = 4.6–10.0×10^7 sperm mL^{-1}). One millilitre of this sperm solution was then mixed with 9 mL of filtered seawater, and the dilution process was repeated, to make 10 mL of low-sperm concentration treatment solution (1 % of the high sperm concentration). Two millilitres of either the high-sperm, low-sperm, or FSW (control) was mixed into each Petri dish and left covered to fertilize for 1 h in a constant temperature cabinet at 22 °C (at which time >50 % of cleaving eggs were beyond the two-cell stage).

Measurements of egg size and larval size

Digital images of eggs were recorded on a dissecting microscope under 45× magnification using PixeLink Capture SE software (PixeLINK, Ottawa, Canada). A random sample of 30 eggs that were not showing any signs of development were measured per treatment using Image-Pro express (version 5.1, Media Cybernetics, Silver Spring, Maryland). This indirect measure of the size distribution of fertilized eggs was necessary because fertilization is determined by the presence of cleavage, which changes the shape of eggs. Hence, the size of fertilized eggs cannot be directly compared to unfertilized controls (Marshall et al. 2002). 'Total egg area', representing the target size of the egg, was defined as the area of the egg including both the ovicell and follicle cells. Thus, 'total egg area' was measured by tracing around the perimeter of the follicle cells. The 'ovicell area' (main energy investment in the egg) was measured by tracing around the intersection of the follicle cells and ovicell. These are standard techniques that have been used in previous studies in this species (Crean and Marshall 2008).

To verify our interpretation that an increase in the size of non-developing eggs represents preferential fertilization of smaller eggs, in six of the trials, fertilized eggs were left to develop into larvae to measure offspring size. Larvae were reared and measured using standard techniques as in previous studies (Crean et al. 2011). Briefly, fertilized eggs were placed in a covered, 100-mL beaker of seawater and left in a constant temperature cabinet at 22 °C. After 12 h, when all viable larvae had hatched, larvae were collected with a pipette, transferred to a specimen container and fixed in buffered formalin. Digital images of 20 larvae in each treatment per trial were recorded as above, and larval body length was measured using Image-Pro express.

Data analysis

Data were analysed using general linear mixed models with restricted maximum likelihood estimation (REML), including 'Treatment' (high sperm, low sperm, and control) as a fixed factor and 'Female' as a random factor. In the analysis of total egg area, ovicell area was included as a co-variate to examine the influence of follicle cell area while accounting for selection acting on ovicell size. A treatment by ovicell interaction term was fitted to the initial model, but was not significant ($F = 0.959$, $p = 0.384$), and so was removed from the final model (Quinn and Keough 2002). Random distribution of model residuals was verified by inspection of residual plots. Analyses were run using JMP (version 10.0.0, SAS Institute).

Results

The size distribution of eggs that were successfully fertilized (measured indirectly as the size of eggs remaining uncleaved) was dependent on the concentration of sperm used in our in vitro fertilization trials. The ovicell area of uncleaved eggs in high sperm concentration treatments was significantly larger than both low-sperm and control treatments ($F = 52.024$, $p < 0.001$; Fig. 1a), meaning that eggs with a smaller ovicell area were selectively fertilized in high sperm concentrations. There was no significant difference between the ovicell size of unfertilized eggs in the low-sperm concentration trials and the control treatment, meaning ovicell area did not influence fertilization success in low-sperm concentration treatments. Female (random factor) accounted for 15.25 % of the total variance in the model. Larvae that hatched from low-sperm concentration treatments were significantly longer than larvae from high sperm concentration treatments ($F = 4.504$, $p = 0.035$,

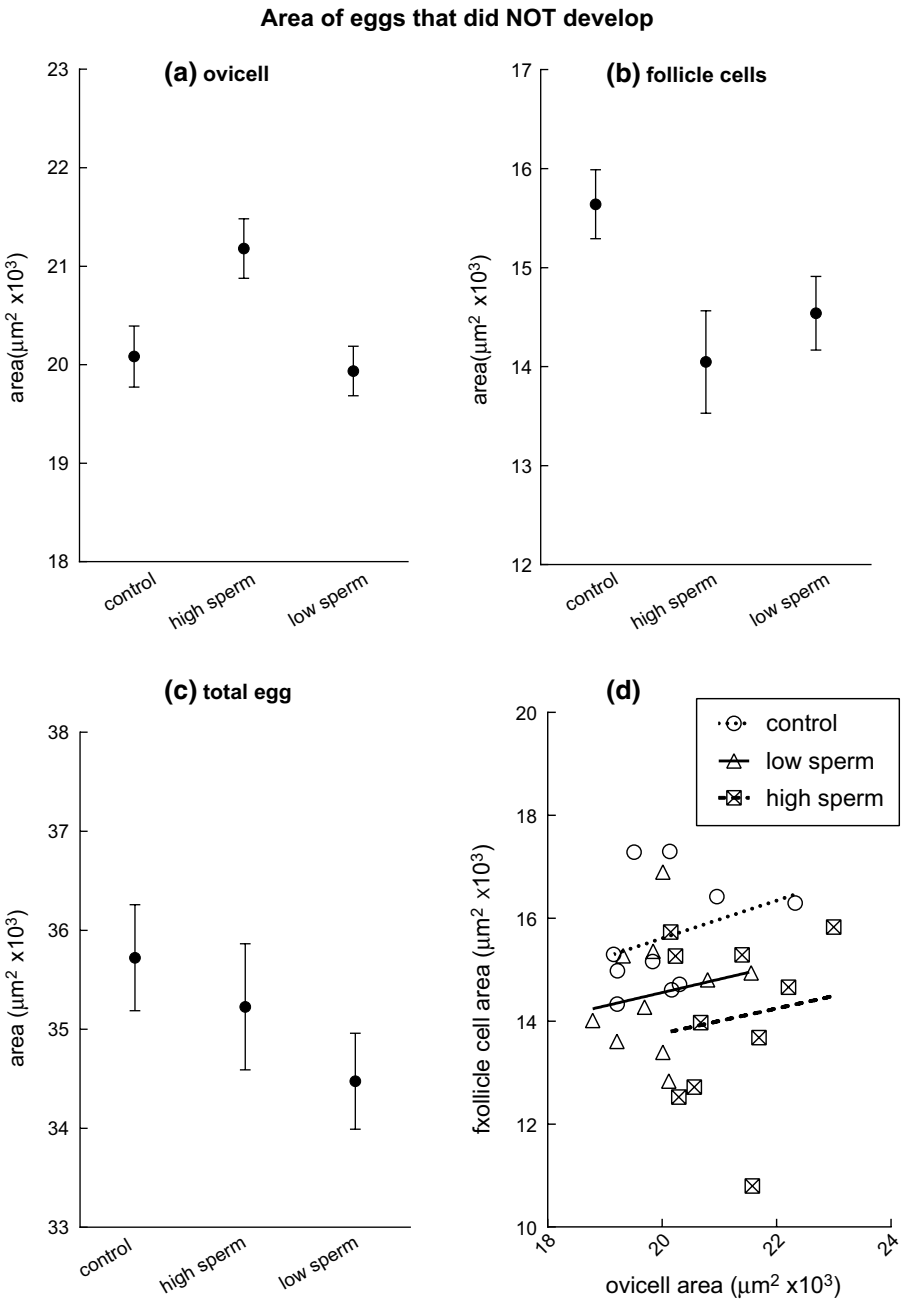

Fig. 1 Average area of eggs showing no development 1 h after initiation of fertilization. **a** Ovicell area, **b** area of follicle cells, **c** total area including follicle cells and ovicell, **d** relationship between average ovicell and follicle cell area across ten replicate trials ($n = 30$ eggs treatment^{-1}). Points show mean ± SE

mean larval length high-sperm treatment = 822.44 µm, mean larval length low-sperm treatment = 834.46 µm).

The area of follicle cells surrounding the ovicell also influenced which eggs were successfully fertilized. Accounting for the influence of ovicell size on fertilization, eggs remaining uncleaved in both high-sperm and low-sperm treatments had a smaller total egg area than controls, meaning eggs with a relatively large follicle cell area were selectively fertilized under both high and low sperm concentrations ($F = 13.690$, $p < 0.001$; Fig. 1b). Ovicell area was positively correlated with total egg area ($\beta = 0.129$, $F = 4.788$, $p = 0.029$; Fig. 1d). Hence, eggs with a larger total area were selectively fertilized in low-sperm concentrations. However, due to selection for smaller ovicells in high-sperm treatments, there was no difference in the total area of eggs in high-sperm and control treatments (Fig. 1c). Female (random factor) accounted for 15.51 % of the total variance in the model.

Discussion

Styela plicata eggs with a large area of follicle cells were more likely to be successfully fertilized in both high and

low sperm concentration fertilization assays. Eggs with a smaller ovicell area were more likely to be successfully fertilized in high sperm concentrations, resulting in smaller larvae hatching from high sperm concentration treatments. We suggest that eggs with a large follicle cell area but small ovicell area are less likely to suffer polyspermy in high sperm concentrations. In contrast, absolutely larger eggs are more likely to be fertilized in low-sperm concentrations. These results demonstrate that in *S. plicata*, follicle cells do not eliminate selection on ovicell size and suggest that follicle cells play different roles in different sperm environments (Levitan 2006).

In low-sperm concentrations, larger follicle cell areas were most likely associated with higher fertilization success because they increased the overall target size of eggs (Podolsky 2002). This fits with previous research that has found ascidian follicle cells assist in sperm aggregation and attraction (Kawamura et al. 1988; Villa and Patricolo 1993). In particular, the follicle cells of *S. plicata* are thought to function as a sperm attractant and to facilitate sperm penetration through the vitelline coat (Villa and Patricolo 2000). In high sperm concentrations, we suggest that large follicle cells increased fertilization success by reducing polyspermy. It has been suggested that accessory structures could help to reduce polyspermy by regulating sperm arrival times or acrosomal processes (Podolsky 2001). Furthermore, the early first block to polyspermy in ascidian eggs results from an enzyme released primarily from follicle cells (Lambert et al. 1997; Lambert 2009). Therefore, an increase in follicle cell area may increase fertilization success in high sperm concentration environments by increasing the speed or efficiency of the initial polyspermy block, or simply by increasing variation in the arrival time of sperm to the ovicell.

Given the multiple benefits to fertilization success of a large follicle cell area, why do we see so much variation in follicle cell area both between and within females? What are the balancing costs limiting the size of follicle cells? Possible constraints on follicle cell size include physical space limitation, genetic links between traits, and energetic constraints. The energetic content of accessory structures is less than that of the ovicell in echinoderms (Bolton et al. 2000), and it is assumed that follicle cells are similarly cheap to produce. However, the production of large follicle cells may still constitute a significant energetic investment, none which can be used by the developing zygote (Podolsky 2004). Therefore, investment in the follicle cells may come at a cost to investment in the ovicell—which has implications for post-settlement success (Marshall and Keough 2008). In addition, changes in follicle cell area may directly influence post-zygotic performance, by altering factors such as egg buoyancy and development time (Villa and Patricolo 1993, 2000). Further experiments are required to disentangle the potential costs of an increase in follicle cell area, particularly how changes in follicle cell area influence offspring performance post-fertilization.

In similar experimental designs to the present study, both Podolsky (2001) and Levitan and Irvine (2001) showed that an increase in the jelly coat area of echinoderm eggs is selected for in sperm-limiting conditions. However, Podolsky (2001) argued that as selection pressures were similar for both the ovicell and jelly coat, and jelly coats are cheaper to produce, selection should favour increased investment in jelly coats. In contrast, Levitan and Irvine (2001) argued that because the unstandardized selection gradient was much steeper for the ovicell, selection should favour increased investment in the ovicell. In our study, we found no selection for ovicell size in sperm-limiting conditions, but instead found that more eggs with smaller ovicells were successfully fertilized in high sperm concentrations. This implies that eggs with larger ovicells became polyspermic, suggesting that sperm are preferentially attracted to the ovicell and that follicle cells do not buffer all selection on ovicell size at fertilization in *S. plicata*.

Overall, these results indicate that both the ovicell and follicle cell area interact with the local sperm environment to influence fertilization success. Eggs with a relatively larger follicle cell area were selectively fertilized in both high and low sperm concentrations, implying that follicle cells can serve multiple functions depending on the environment. The fertilization environment also influenced the distribution of ovicell sizes that were successfully fertilized, which resulted in larger larvae being produced in low-sperm concentration conditions. Hence, broadcast-spawning mothers not only face the challenge of potentially opposing pre- and post-zygotic selection pressures on egg size, but also face the challenge of potentially opposing selection pressures on different components of their eggs. How a mother allocates her resources to the main ovicell versus egg accessory structures is likely to depend on the relative strengths of selective pressures, the reliability of environmental cues, and the costs of plasticity (Burgess and Marshall 2014).

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