***In vitro* fertilization experiments using sockeye salmon reveal that bigger eggs are more fertilizable under sperm limitation**

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Although theory and widespread evidence show that the evolution of egg size is driven primarily by offspring and maternal fitness demands, an additional explanation invokes sperm limitation as a selective force that could also influence egg size optima. Levitan proposed that constraints from gamete encounter in external fertilization environments could select for enlargement of ova to increase the physical size of the fertilization target. We test this theory using *in vitro* fertilization experiments in an externally fertilizing fish. Sockeye salmon (*Onchorhynchus nerka*) females show considerable between-individual variation in ovum size, and we explored the consequences of this natural variation for the fertilization success of individual eggs under conditions of sperm limitation. By engineering consistent conditions where *in vitro* fertilization rate was always intermediate, we were able to compare the sizes of fertilized and unfertilized eggs across 20 fertilization replicates. After controlling for any changes in volume through incubation, results showed that successfully fertilized eggs were significantly larger than the eggs that failed to achieve fertilization. Under conditions without sperm limitation, fertility was unaffected by egg size. Our findings therefore support Levitan's theory, demonstrating empirically that some element of egg size variation could be selected by fertilization demands under sperm limitation. However, further research on sperm limitation in natural spawnings is required to assess the selective importance of these results.

Keywords: sperm; ova; fertilization; salmon; gamete

1. INTRODUCTION

Parker *et al.* (1972) theorized that the evolution of anisogamy occurred via disruptive selection, within a fixed evolutionary strategy, for either competitive or nutritive gametes. Competition for gamete fusions favoured a strategy that maximized sperm number (at the expense of size), while selection for zygote fitness favoured a strategy that increased egg size (at the expense of numbers; Parker 1982). There is good empirical evidence that increased sperm number bestows benefits in sperm competition (e.g. Gage & Morrow 2003). Similarly, widespread evidence from experimental and correlative studies shows that variation in ova/propagule size is directly related to individual offspring fitness (e.g. Burgner 1998; Einum *et al.* 2004), and that investment in egg size must be balanced against egg number to maximize maternal fitness (e.g. Smith & Fretwell 1974; Einum & Fleming 2000). Variation in ovum size can therefore be primarily explained through offspring and maternal fitness optima.

More recently, a theory for the evolution of egg size has been suggested, which is additional to the established influences on offspring fitness. Levitan (1993, 1996) proposed that increased ovum size evolved under external

fertilization to create a larger 'target' for fertilizations. Levitan hypothesized that, in an external fertilization environment, sperm limitation could be a selective force on gamete evolution, with egg size selected to increase above what was necessary for post-zygotic survival in order to enlarge the size of the fertilization target. In Levitan's (1996) model, sperm are selected to become tiny and numerous, not as a result of sperm competition, but due to selection for the ability to locate ova in a diffuse fertilization medium.

There is good evidence that sperm limitation occurs naturally in externally fertilizing organisms (e.g. Pennington 1985; Levitan & Petersen 1995), including in fishes that release gametes in close proximity (Petersen *et al.* 1992). The production of eggs that do not get fertilized could therefore be a selective cost for females, logically leading to the theory that eggs may be under selection to maximize their own fertilization probabilities. In support of this theory, comparative studies across three *Strongylocentrotus* sea urchin species revealed significant correlations between egg cross-sectional area and concentration of sperm needed to fertilize eggs under both laboratory (Levitan 1993) and field (Levitan 1998) conditions. Levitan also found evidence for the effect of egg size on fertilization success within urchin species, and within clutches within species (Levitan 1998). Thus, there is empirical evidence that egg sizes of

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broadcast-spawning marine invertebrates could increase as a result of selection for increasing the probability of fertilization under sperm limitation.

Levitan's theory for the evolution of egg size has only been tested in broadcast-spawning marine invertebrates. Here we test his theory in an externally fertilizing fish where males and females spawn in closer association. Sockeye salmon (*Oncorhynchus nerka*) exhibit significant variation in egg size among females (e.g. Burgner 1998), providing an excellent opportunity for an experimental test of Levitan's theory. The adaptive significance of egg size variation in salmon in relation to the optimal balance between offspring fitness and number (Smith & Fretwell 1974) under varying ecological and evolutionary constraints has received considerable attention and is well established (Fleming & Gross 1990; reviewed by Einum *et al.* 2004). An experimental study testing the Smith & Fretwell (1974) model for egg size: number variation in Atlantic salmon demonstrated convincing support for egg size optima that maximize individual maternal fitness through total offspring survival. We therefore recognize that variation in egg size within salmon can be primarily explained through offspring and maternal fitness optima. However, no studies have explored fish egg size variation with regard to 'fertilizability'. Sockeye salmon provide an excellent system in which to test the relevance of egg size for fertilizability: there is significant natural variance in egg size, and *in vitro* fertilization techniques for conducting controlled fertilization experiments in the natural gametic microenvironment are established (Hoysak *et al.* 2004); these characteristics allow Levitan's (1993, 1996) theory to be experimentally tested in a fish model.

2. MATERIAL AND METHODS

(a) Fish sources and gamete collection

Experimental work was carried out at the Fisheries and Oceans Canada Fulton River Salmon Enhancement Project in British Columbia, Canada. Twenty Males and 15 females in full breeding condition with free-running milt or eggs were stripped of gametes, taking care not to allow contamination with water, urine or mucus. Eggs from all 15 females were pooled, so that fertilization trials contained a range of egg sizes, and any individual female fertility effects were randomly and evenly spread across all trials. To control for any male time-since-strip effects, milt was stripped from each male just prior to each fertilization trial, so that the time from strip to fertilization never exceeded 6 min. Eggs were stored at approximately 3°C in ovarian fluid without exposure to water, and all fertilization trials were completed within 2.5 hours of stripping. Studies of other salmonids (e.g. Holcomb *et al.* 2005) and our own preliminary tests show that this period and these conditions do not result in egg or sperm fertility changes. Importantly, the experimental comparisons followed a paired design where each of 20 individual fertilization trials was designed to generate eggs that were both successful and unsuccessful in achieving fertilization, and therefore comparisons could be made within each trial. Thus, potential confounds from effects such as time since strip were eliminated. Moreover, although we employ a paired experimental design, because the eggs are pooled from multiple females, to be conservative, we employ independent *t*-tests for analysis.

(b) In vitro fertilization trials

To examine the effect of egg size on fertilization success, we ran 20 *in vitro* fertilization trial replicates using milt from 20 different males, and under sperm limited conditions. For each replicate, 20 ml of eggs (approx. 150 eggs) from the egg pool were fertilized with 5 µl of milt in 500 ml of Fulton river water at approximately 6°C. This protocol ensured intermediate fertilization success (approx. 20 to approx. 80%) of each egg batch. After each fertilization trial, eggs were left to stand for 5 min before being carefully placed in small containers and transferred to Heath tray incubators. At this stage, embryogenesis is sensitive to physical movement, so we did not handle or measure the eggs until the embryo could be seen, which takes over 10 days of incubation at approximately 6°C.

(c) Egg scoring and measurement

Eggs were placed in 5 per cent acetic solution for 15 min so that the developing embryo in fertilized eggs could be observed (Hoysak & Liley 2001). Subsequently, 30 fertilized and 30 unfertilized eggs were randomly selected and measured in every trial using an eye-piece graticule at ×8 magnification on a Nikon dissecting microscope. Two measures of diameter were made for each egg (along the north-south and east-west axes). Eggs are spherical, so that the mean of the two diameter measures was used to calculate surface area of the egg ($=r^2(4\pi)$).

(d) Control treatments

To control for any changes in egg size associated with activation in water, fertilization and exposure to acetic acid, two control treatments were conducted in which eggs were activated and incubated identically to the experimental groups, but one group was treated so that full fertility could be achieved, and the other group was not fertilized. Additionally, the first control importantly checked that fertilization success without sperm limitation was not a function of egg size. In the first control, a sample of eggs from the pool of mixed eggs was fertilized in river water with 20 µl of sperm, which we know is sufficient milt to ensure 100 per cent fertilization under these conditions. In a second control group, a subsample of eggs (again from the pool of mixed eggs) were simply activated in Fulton river water without milt to create zero fertilization. Subsamples of eggs from both groups were measured within 5 min of fertilization or activation, taking extra care not to physically disrupt embryogenesis in the fertilized treatment, and then again 10 days after 'incubation' as per the experimental replicates. It is important to note that egg surface area does not change over the first 5 min of activation for fertilized or unfertilized eggs (paired $t_{19} = -0.38$, $p = 0.71$ and $t_{19} = -0.5$, $p = 0.62$); eggs in both groups show highly significant correlations between egg surface area measured in the pre-activated state compared with measurements made between 3 and 5 min after activation in river water (fertilized: $r^2 = 0.9$, $p < 0.0001$, $n = 20$; unfertilized: $r^2 = 0.94$, $p < 0.0001$, $n = 20$). Control eggs were held in individual containers on the Heath trays so that they could be identified. Accordingly, we were able to use any changes in egg size through activation, fertilization or acetic acid treatment to back-calculate the size of fertilized or non-fertilized eggs at the point of sperm limitation in the experiment. Additionally, for the 'fertilized' control we scored fertilization success of all eggs, to check for an influence of egg size upon fertility in the absence of sperm limitation.

3. RESULTS

In the first control using 20 μ l milt aliquots, 100 per cent fertilization success was achieved throughout. For both groups of eggs, there were highly significant relationships between egg surface area within 5 min of activation, and surface area after 10 days of incubation (unfertilized eggs: $r^2=0.89$, $n=34$, fertilized eggs: $r^2=0.96$, $n=27$; see figure 1). Paired comparisons of changes in cell surface area over 10 days of incubation showed no significant change in egg surface area from 5 min post-activation to acetic acid scoring 10 days later for fertilized eggs (paired $t_{26}=1.178$, $p=0.25$). However, unfertilized eggs showed a significant approximately 5 per cent increase in egg surface area from 5 min post-activation to embryo scoring 10 days later ($t_{33}=-7.455$, $p<0.001$). This difference was not attributable to the effect of acetic acid, as there was no significant difference between egg surface area immediately before and after acetic acid treatment (paired $t_{33}=-2.016$, $p=0.52$).

We therefore determined the relationship between unfertilized egg surface area at 10 days and that at time of activation, and found across 34 ova that 89 per cent of the variation in egg size could be explained by this consistent swelling ($r^2=0.89$, $F_{1,32}=265.79$, $n=34$, $p<0.001$). We could therefore accurately correct for changes in surface area among unfertilized eggs in the experimental trials by back-calculating the egg surface area at activation using the equation derived from this regression of control egg size at 5 min post-activation versus control egg size after 10 days of incubation: Predicted initial egg surface area $=(0.891 \times \text{egg surface area at 10 days}) + 4.71$. Although our experiment is a paired design, the eggs in each trial are mixed from different females, therefore we analyse any differences using more conservative independent t -tests.

Having corrected for changes in unfertilized ova, we were able to compare the surface areas of eggs that were fertilized versus eggs that were not fertilized across 20 replicate *in vitro* fertilization trials. Under sperm-limiting conditions, we found that eggs that were successfully fertilized had significantly larger surface areas than eggs that failed to achieve fertilization (independent $t_{38}=8.449$, $p<0.001$). Fertilized eggs had a surface area that was approximately 7 per cent greater than unfertilized eggs (figure 2). This difference could not have been an artefact of our correction for the swelling of unfertilized eggs because the surface area of fertilized eggs was also significantly greater than the uncorrected surface area of unfertilized eggs, although the magnitude of the difference was reduced (independent $t_{38}=2.738$, $p<0.01$).

4. DISCUSSION

When we experimentally created fertilization environments with sperm limitation, salmon eggs with a greater cell surface area were preferentially fertilized. Our experiment controlled for changes in unfertilized egg surface area that occurred over the 10-day incubation period. Furthermore, when we removed sperm limitation to achieve 100 per cent fertilization success, eggs of all sizes were fertilized equally; thus, our results are unlikely to be an effect of egg size upon embryonic development or survival. These findings therefore add experimental support to Levitan's (1993, 1996) theory that the

Figure 1. Changes in control egg surface area over the 10-day incubation period. Fertilized eggs (filled circles and line of best fit) showed no change in surface area (paired $t_{26}=1.178$, $p=0.25$), but unfertilized egg surface area (open circles and line of best fit) increased by approximately 5% (paired $t_{33}=-7.455$, $p<0.001$). We therefore back-calculated unfertilized egg measurements at 10 days to quantify predicted surface areas at activation/fertilization using the regression equation $Y=0.891X+4.71$ ($r^2=0.89$). Dashed line plots relationship of unity (=no change).

Figure 2. Mean ($\pm$ s.e.) cell surface area is significantly greater for fertilized than unfertilized eggs under sperm limitation ($p<0.001$). $N=20$ paired trials, with 30 fertilized and 30 unfertilized eggs measured in each trial.

evolution of increased egg size can be favoured when sperm is limited. In a similar manner to broadcast-spawning marine urchins (e.g. Levitan 1993, 1998), we found that increased egg size bestows a fertilization advantage when gamete fusion is limited by sperm density.

It is important to consider these findings in relation to the level of selection from sperm limitation in natural spawnings. A review across marine free spawners concluded that natural fertility rates are usually over 85 per cent (Yund 2000). Probably the best-studied pair-spawning fish is the bluehead wrasse (*Thalassoma bifasciata*), in which measurement of 810 natural spawnings revealed a mean fertilization success of 95 per cent with a range from 83 to 100 per cent. Less information is available for salmonids, where eggs are buried under gravel after spawning. However, in studies measuring fertilization success of spawnings in natural or experimental streams, similar fertility rates are found, e.g. 87–95% in Atlantic salmon (*Salmo salar*; de Gaudemar *et al.* 2000), and 80–85% fertility averages in Chinook salmon (*Onchorhynchus tshawytscha*; Berejikian & Tezak 2005). In a study of

sockeye salmon spawning dynamics, Mathison (1962) found fertility to be generally high in 'normal' spawnings (98.4%), with fertility declining as the experimental operational sex ratio became more female biased (81.6–97.5%). It is important to note that these measures cannot distinguish between unfertilized fertilized eggs but suffered early embryo arrest. Thus, the few studies of natural salmonid spawnings indicate that natural external fertilization rates are generally high, so the species-specific applicability of our findings remains under question. Nevertheless, our results do indicate that, in those fish species where sperm limitation does occur, egg size could be selected to improve fertilizability. Data from a greater number of redds and species across the full range of population structures encountered on the redds are required to assess fully the importance of sperm limitation in natural salmon spawnings, and other fish species.

Information from *in vitro* fertilization dynamics within salmonids and their gamete biology suggest that sperm limitation could be a selective force under certain conditions. Salmonid males can produce huge numbers of gametes (Gage *et al.* 1995), but sperm lifespan after activation in freshwater is a brief matter of seconds, due to osmotic stress (e.g. Billard & Cosson 1992; Gage *et al.* 1995; Robertson 1996; Hoysak & Liley 2001; Liley *et al.* 2002), leading one author to claim that salmonid sperm are 'maladapted' to freshwater spawning (Huxley 1930). Non-synchronous gamete release could therefore result in limited fertilization rates. *In vitro* sperm competition experiments in Atlantic and sockeye salmon show that a 2 second delay in the introduction of one male's milt to the fertilization set creates significant fertilization losses for that male in two-male sperm competitions (Hoysak *et al.* 2004; Yeates *et al.* 2007), demonstrating the rapid rates of sperm:egg association in salmon. In addition to the constraints set by external fertilization in freshwater and salmonid gamete biology, sperm limitation could also potentially occur in depleted salmonid populations where older, dominant males are in the minority. In territorial reef fishes, the most preferred dominant males gain more spawning, but individual females suffer reduced fertilization success as a result of sperm depletion in these preferred males (Marconato *et al.* 1995; Warner *et al.* 1995). Similar studies of mate choice and population age structures are required for salmonids, especially where populations are severely depleted.

Salmon sperm gain entry to the ovum via a single micropyle (Yanagimachi *et al.* 1992). This situation therefore differs from many broadcast-spawning invertebrates, because the fertilization target is not just the total egg surface membrane, but a single entry point on that surface. Despite micropylar entry, the total egg itself may represent a target for sperm if chemicals released with, and on, the eggs influence sperm motility and fertility. In fishes, ovarian fluid, a fluid that is released simultaneously with eggs, is known to increase both sperm viability (Scott & Baynes 1980; Lahnsteiner *et al.* 1997; Hayakawa & Munechara 1998; Turner & Montgomerie 2002) and fertilization success (Litvak & Trippel 1998), with evidence for differential effects on different males' sperm motility (Rosengrave *et al.* 2008). If ovarian fluid is bound to the egg membrane on release into the freshwater fertilization environment, bigger eggs may

carry a larger ovarian fluid load, and therefore be more effective at encouraging sperm towards the micropyle.

Finally, it is important to consider our experimental findings in the light of the well-known relationship between salmonid egg size and offspring fitness (e.g. Einum & Fleming 2000). In sockeye salmon, there is a positive correlation between the size of three-month-old fry and egg weight (Burgner 1998). Similarly, we have analysed published data on egg volume and dry weight across nine populations of sockeye salmon from the Fraser river drainage system (see table 6 in Burgner 1998). Averaging 20 eggs per population, there is a significant linear relationship between volume and dry weight: dry weight = $-26.74 + 0.74 \times \text{egg volume}$, ($r^2 = 0.85$). Thus, larger eggs carry greater material density, and are not simply increased through aqueous volume, supporting a primary function of egg size to be for providing resources for individual offspring, and ultimately maternal, fitness (Einum & Fleming 2000).

Fish were netted, handled and stripped according to welfare standards maintained by Fisheries and Oceans, Canada.

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