RESEARCH ARTICLE

Fertilisation is not a new beginning: sperm environment affects offspring developmental success

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SUMMARY

For organisms with complex life histories, the direction and magnitude of phenotypic links among life-history stages can have important ecological and evolutionary effects. While the phenotypic links between mothers and offspring, as well as between larvae and adults, are well recognised, the links between sperm phenotype and offspring phenotype have been less well explored. Here, we used a split-clutch/split-ejaculate design to examine whether the environment that sperm experience affects the subsequent performance of larvae in the broadcast spawning marine invertebrate *Galeolaria gemineoa*. The environment that sperm experienced affected the developmental success of larvae sired by these sperm; larvae sired by sperm that experienced low salinities had poorer developmental success than larvae sired by sperm that experienced a normal salinity. When we explored the interactive effects of the sperm environment and the larval environment with an orthogonal design, we found an interaction; when sperm and larvae experienced the same environment, performance was generally higher than when the sperm and larval environments differed. These effects could be due to selection on specific sperm phenotypes, phenotypic modification of the sperm or both. Together, our results challenge the traditional notion that sperm are merely transporters of genetic material; instead, significant covariance between sperm and offspring phenotypes exists. Our study adds to a growing list that demonstrates that fertilisation does have a homogenising effect on the phenotype of the zygote, and that events before fertilisation during the gamete phase can carry through to affect performance in later life-history stages.

Key words: complex life cycles, life-history theory, phenotypic plasticity, stress.

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INTRODUCTION

The life-history stages of organisms with complex life cycles are strongly linked (Marshall and Morgan, 2011; Schluter et al., 1991). From sponges to vertebrates, the phenotype expressed in one life-history stage depends in part on the phenotype and environment of the preceding stage (Beckerman et al., 2002; Pechenik, 2006). These phenotypic links among life-history stages have a number of important evolutionary and ecological consequences. From an evolutionary perspective, these links mean that the phenotype of any one stage is unlikely to be a product of selection pressures on that stage alone (Schluter et al., 1991), such that the optimal phenotype for that stage may be evolutionarily inaccessible. Viewed in this light, the phenotype expressed in one life-history stage may be an awkward compromise between its fitness benefits in that stage and its fitness costs in another stage (Arnold, 1992; Crean et al., 2011; Morgan and Anastasia, 2008). From an ecological perspective, phenotypic links among life-history stages mean that the performance of individuals within a population is unlikely to be driven by current conditions alone; rather, the environments that those individuals experienced in earlier life-history stages may determine some proportion of their performance (Pechenik, 2006). For example, adults living in identical habitats may differ dramatically in performance because some individuals experienced stress as larvae (Hamilton et al., 2008; Pechenik et al., 2001). This presents a major challenge to population ecologists – events in very different, potentially distant habitats can influence the population

dynamics of an adult population *via* these phenotypic links (Marshall et al., 2010). An integration of the environmental influences on different life-history stages, and the phenotypic links among them, should therefore lead to a better understanding of the population dynamics of organisms with complex life cycles (Marshall and Morgan, 2011).

Maternal effects are one of the most obvious sources of phenotypic variation (Mousseau and Fox, 1998; Uller, 2008). Across many taxa, the environment that mothers experience can affect the phenotype of their offspring for the rest of their lives (Fox and Czesak, 2000; Marshall and Uller, 2007). Variability in temperature, pollution, abundance of predators and competitors during egg laying, gestation and embryonic development can all result in profound and persistent changes in the phenotype of the subsequent offspring (Bergeron et al., 2011; Marshall and Uller, 2007). Environmental variation during the larval stage can also carry through to affect the phenotype of the adult life-history stage – indeed, despite the massive tissue reorganisation associated with metamorphosis, it does not always represent a 'new beginning' for the metamorphosed individual (Pechenik, 2006). In a range of taxa, different larval environments can lead to very different larval and adult phenotypes. For example, exposure to predators can induce the expression of predation-resistant larval phenotypes in tadpoles; these predator-resistant tadpoles metamorphose into longer-legged frogs (Relyea, 2001). Thus, the maternal, the egg, the embryonic and the larval environment can all affect an organism's subsequent

phenotype. Interestingly, the environment that sperm experience prior to fertilising an egg has rarely been considered as a source of phenotypic variation in offspring. While this dearth of studies on sperm environmental effects makes sense at first glance – sperm are thought to be simple conveyors of genetic information – there is increasing evidence that the contribution of sperm to the phenotype of their offspring extends beyond their genes alone (Bonduriansky and Day, 2009).

The paternal environment can induce epigenetic changes in sperm that can be stably transmitted to offspring (e.g. de Boer et al., 2010; Flanagan et al., 2006; Jablonka and Raz, 2009; Ng et al., 2010). These studies have largely been restricted to manipulating the paternal environment and determining whether this manipulation influences the offspring phenotype (e.g. Bonduriansky and Head, 2007) (but see Crean et al., 2012). What is less well understood is how variation in the sperm environment once those sperm have been released from the male affects the phenotype of their offspring. In internally fertilising species, the post-ejaculate environment is the female reproductive tract: an environment that, due to homeostasis, should be relatively constant such that the experience of different sperm is relatively similar. In externally fertilising species (such as most fish, amphibians and marine invertebrates), however, sperm are shed into a highly variable, uncontrolled environment where individual spermatozoa, both among and within ejaculates, can experience vastly different conditions. For example, externally shed sperm can be exposed to waterborne pollutants (Hollows et al., 2007), variable thermal regimes (Bownds et al., 2010) and very different hydrodynamic conditions (Mead and Denny, 1995). Whether this massive variation in the sperm environment generates differences in the phenotype of the offspring these sperm go on produce remains largely unexplored.

Here, we manipulated the environment that sperm experience after release but before fertilisation, and examined the consequences of this sperm experience for subsequent offspring performance in a broadcast spawning marine invertebrate. We used a split-clutch/split-ejaculate design (Evans and Marshall, 2005) to control for any confounding paternal genetic effects or maternal effects. We focused on the broadcast spawning, serpulid polychaete *Galeolaria gemineoa* Halt, Kupriyanova, Cooper & Rouse 2009 – a species with a brief (6 days) planktotrophic larval period. This species is commonly found on the coast of Australia, living in the intertidal zone (Fitzpatrick et al., 2008; Ross and Bidwell, 2001). *Galeolaria* are ideal for studies investigating gametes because they produce mature gametes year round, under field and laboratory conditions (Andrews and Anderson, 1962; Marsden and Anderson, 1981). While studies of fertilisation and development have been done on the southern species *Galeolaria caespitosa*, here we worked on *Galeolaria gemineoa* – the northern species with an identical life history. Adult *Galeolaria* often occur in estuaries that are regularly exposed to varying water salinities, and can efficiently regulate their osmolarity in the range ~21–37 p.p.t. (Tait et al., 1981). However, early life stages and gametes of *Galeolaria* are sensitive to salinity extremes: in the laboratory, embryos developing in a salinity of 21 p.p.t. suffer 100% mortality (cf. 10% mortality in normal salinity) (Tait et al., 1984).

MATERIALS AND METHODS
Overview and general methods

Our overall approach was to expose a portion of a male's ejaculate to a reduced level of salinity (such that this species would regularly encounter after rainfall), and another portion of the same ejaculate to a normal salinity level for a short period. Sperm from both treatments were then diluted with an appropriate salinity to return to (or maintain) a normal salinity level. Eggs and sperm were then mixed so that fertilisation could take place at a normal salinity level, thereby precluding any potentially confounding influence of the egg or fertilisation environment as low salinity during fertilisation is known to reduce fertilisation success in marine invertebrates (Allen and Pechenik, 2010). We then measured the developmental success (the proportion of zygotes that developed into larvae). We subsequently explored whether offspring sired by sperm that had been exposed to lower salinity were more resistant to lower salinity themselves.

Mature *G. gemineoa* were collected from The Spit, QLD (27°S, 151°E) for experiments 1 and 2, and from both The Spit and Burleigh Heads, QLD (28°S, 153°E) for experiment 3, during the period of September 2010 to April 2011. Both sites experience typical salinities of 35–37 p.p.t., but following rainfall events salinity can drop as low as 22 p.p.t. in the area adjacent to adult populations, while salinities remain relatively constant a few hundred meters away (D.J.M., unpublished data).

Adult worms were maintained at ambient temperature in insulated aquaria, with constant aeration in seawater between 35 and 37 p.p.t. collected from the study site. Gametes were collected with standard techniques (Marshall and Evans, 2005a; Marshall and Evans, 2005b). Briefly, mature adults were removed from their calcareous tubes using tweezers, and placed into a 15 ml Petri dish with 3 ml of filtered sea water (FSW) for females and 1 ml of FSW for males. For all of our experiments, we conducted fertilisation in a normal (37 p.p.t.) salinity; similarly, eggs were only exposed to a normal salinity.

Experiment 1: effect of sperm environment on developmental success

In this experiment, we were interested in whether the sperm environment affected the subsequent developmental success of larvae. To manipulate the environment that sperm experienced, we exposed a highly concentrated 0.1 ml solution of sperm to 4 ml of artificial sea water (ASW; made using commercial seawater aquarium salt and reverse osmosis freshwater) with a salinity of 25, 30 or 37 p.p.t. for 15 min. From each solution we then collected a 0.5 ml sperm sample and returned this sample to a salinity of 37 p.p.t. by exposing it to 0.5 ml of appropriately hypersaline or normal salinity ASW (depending on the treatment). Care was taken to mix the solution well during each stage of salinity manipulation. This approach mimics the likely experience of gametes in the field when they are shed into a low salinity environment driven by freshwater run-off before being transported to a more normal salinity environment in the well-mixed open coast.

To conduct our *in vitro* fertilisations, we exposed the eggs of a single female to the treated sperm for 15 min, after which the eggs were rinsed of sperm and an additional 10 ml of FSW was added and the eggs were left to develop. The fertilisation success of 50 eggs per sample was scored 2 h after the initial exposure to sperm and we classed eggs as 'fertilised' if they had begun cell cleavage (99% of fertilised eggs were at the 2–4 cell stage by this time). Control batches of eggs that have not been exposed to sperm do not show spontaneous cell cleavage in our experience with this species. Sperm concentrations were kept relatively constant throughout our experiments (~10^6 sperm ml^{-1}) and fertilisation rates were similar across all of our experiments at ~50%. In pilot studies, we found no significant effect of sperm exposure to hyposalinity on subsequent fertilisation success ($F_{1,12}$=0.003, P=0.958), nor was there any indication of such an effect (mean ± s.e.m. fertilisation success for low salinity: 56.43±5.7; normal salinity: 56.00±5.2).

To estimate larval developmental success, we collected 25 fertilised embryos from each treatment (all embryos were the same age) and placed each into their own 90 mm Petri dish with 10 ml of FSW. The embryos were then kept in a constant temperature cabinet (25°C) for 12 h and left to develop into trochophores (the first larval stage). Using a dissecting microscope, we then scored the number of trochophores alive in each Petri dish before returning the Petri dishes to the temperature cabinet for a further 12 h, whereupon the number of larvae that were still alive was again assessed. We explored the effects of our sperm treatment over time because we were interested in determining the persistence of any effects that we observed. For any one experimental run, we created between one and four subreplicates of each treatment (depending on the size and therefore fecundity of the focal female), and we conducted seven experimental runs. Across all of our experiments, each run consisted of eggs from a single female with a single male, and each run used different individuals. Within each run, we used multiple subreplicates to increase the precision of our estimates of the effects but used the mean value for each run (the appropriate unit of replication) in our analysis (Quinn and Keough, 2002).

Experiment 2: effect of larval environment on developmental success

In this experiment, we were interested in whether a hyposaline larval environment reduced developmental success. In the following experiments, we focused all of our replication on just two environments, 25 and 37 p.p.t., as the strongest effects are to be expected between the two extremes. To examine the effect of the larval environment on developmental success, we created fertilised embryos as described above, but in this experiment sperm were not exposed to a hyposaline environment; rather, they were all exposed to normal salinity conditions. After fertilisation, two groups of 25 fertilised eggs were removed: one group of 25 fertilised eggs was placed in 10 ml of salinity solution of 25 p.p.t.; the other group of 25 eggs was placed into a 37 p.p.t. salinity solution. The embryos were then allowed to develop as described above, and developmental success was scored after 12 and 24 h. We used only a single replicate per run for this experiment and conducted nine experimental runs.

Experiment 3: effect of sperm and larval environment on developmental success

We found effects of both the sperm and the larval environment on subsequent offspring performance (see Results), and in this experiment we exposed both sperm and larvae to hyposaline or normal salinity conditions (again, we only used two salinity levels). In this experiment, all of our methods were identical to those in experiments 1 and 2 except that the salinity treatments were applied orthogonally across both life-history stages while fertilisation occurred in normal salinity only. In this experiment, we were only able to assess developmental success 24 h after fertilisation. We replicated this experiment using adults from two collection sites (seven runs from Burleigh heads and nine runs from The Spit) with between three and four subreplicates of each treatment within each run. Again, the mean of these subreplicates for each run was the unit of replication that was analysed.

Data analysis

All analyses were carried out in Systat version 13. For experiment 1, we used a repeated measures ANCOVA to examine the effects of our salinity treatments (continuous factor) and time (fixed factor): for experiments 2 and 3, we used a mixed model ANOVA. Where appropriate, we partitioned variance associated with different

Table 1. Effect of exposure of sperm to low salinity on subsequent developmental success of *Galeolaria gemineoa* embryos and larvae over time

Source	d.f.	MS	F	P
Between subject				
Sperm salinity environment	1	0.117	5.47	0.036
Run	6	0.034	1.59	0.228
Error	13	0.021		
Within subject				
Time	1	0.022	0.57	0.466
Time × sperm salinity environment	1	0.008	2.17	0.165
Time × run	6	0.006	1.76	0.186
Error	13	0.004		

Repeated measures ANCOVA for salinity effects. Success was measured as a proportion. Sperm salinity was a continuous factor, experimental run was a random effect. The survival of larvae was estimated 12 and 24 h after fertilisation, hence the time effect. Note that model is reduced after testing for non-significant random interactions.

runs [random factor: either run or run(site) depending on the experiment] and different populations (random factor: site). For experiment 1, there was no effect of time × treatment × run, nor was there an interaction between treatment × run, and so these interaction terms were removed from the final model (Quinn and Keough, 2002). For experiment 3, there was no effect of site × larval treatment × sperm, and none of the lower order random interactions was significant, so the model was reduced accordingly (Quinn and Keough, 2002).

RESULTS
Experiment 1: effect of sperm environment on developmental success

Exposure of sperm to low salinity resulted in the reduced survival of larvae 12 and 24 h post-fertilisation (Table 1). After 12 h, around 50% of zygotes sired by sperm exposed to the lowest salinity had successfully developed into larvae compared with around 70% of zygotes sired by sperm exposed to normal salinity (Fig. 1). After
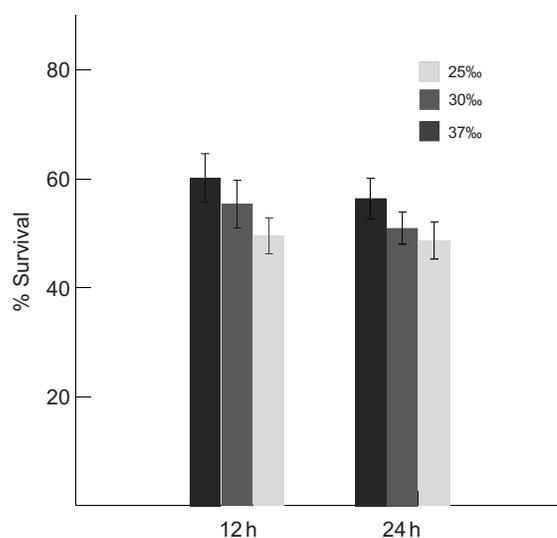

Fig. 1. Effect of the salinity that *Galeolaria gemineoa* sperm experience prior to fertilisation on the developmental success of subsequent larvae. Note that, for all treatments, fertilisation was conducted at normal salinity. Bars show the mean ± s.e.m. percentage of fertilised eggs that successfully develop into larvae after 12 and 24 h for seven experimental runs (replicates).

Table 2. Effect of the larval salinity environment on the developmental success of *G. gemineoa*

Source	d.f.	MS	F	P
Between subject				
Larval salinity environment	1	0.046	5.39	0.049
Run	8	0.039	4.61	0.022
Error	8	0.008		
Within subject				
Time	1	0.037	23.74	0.001
Time × larval salinity environment	1	0.000	0.05	0.826
Time × Run	8	0.002	1.60	0.260
Error	8	0.002		

Repeated measures ANOVA for salinity effects. Success was measured as a proportion. Larval salinity was a categorical fixed factor, experimental run was a random effect. The survival of larvae was estimated 12 and 24 h after fertilisation, hence the time effect. Note that model is reduced after testing for non-significant random interactions.

Table 3. Effect of sperm environment and larval environment on the developmental success of *G. gemineoa*

Source	d.f.	MS	F	P
Site	1	2742	83.36	<0.001
Sperm salinity environment	1	22	0.69	0.410
Larval salinity environment	1	37	1.14	0.292
Larval salinity environment × sperm salinity environment	1	178	5.42	0.024
Run (site)	14	425	12.92	<0.001
Error	45	32		

ANOVA for salinity effects. Success was measured as a proportion. Sperm salinity was a categorical fixed factor, experimental run was a random effect. We repeated this experiment using individuals collected at two sites. Note that model is reduced after testing for non-significant random interactions.

24 h, the proportion of zygotes that had developed into larvae in all treatments was slightly lower but the effects of sperm exposure persisted (Fig. 1). Casual observations suggested that very few embryos died during the early phases (4–8 cell stage) of development; rather, most of the mortality occurred later.

Experiment 2: effect of larval environment on developmental success

The larval environment had a minor but significant effect on larval developmental success (Table 2). Larvae developing in the lower salinity environment suffered a slight reduction in developmental success across both time periods relative to larvae developing in the normal salinity environment (Fig. 2).

Experiment 3: effect of sperm and larval environment on developmental success

The sperm environment and the larval environment interacted to affect larval developmental success (Table 3). Larvae sired by sperm that had been exposed to lower salinity performed better in low salinity themselves relative to larvae sired by sperm from a normal salinity environment (Fig. 3). In other words, developmental success was highest when the larval environment matched the environment that sperm experienced. Simple main effects tests revealed that this matching effect was not symmetrical, however. In the normal salinity larval environment, there was a significant difference in the developmental success of larvae sired by sperm that had experienced different environments ($F_{1,15}$=7.97, P=0.013), but in the hyposaline larval environment, there was no significant difference ($F_{1,15}$=1.52, P=0.236).

DISCUSSION

We found that the environment that sperm experienced affected the subsequent performance of offspring that those sperm sired. Generally, the survival of larvae was lower if their parental sperm had been exposed to low salinity but this effect depended on the environment the larvae experienced themselves. Larvae survived
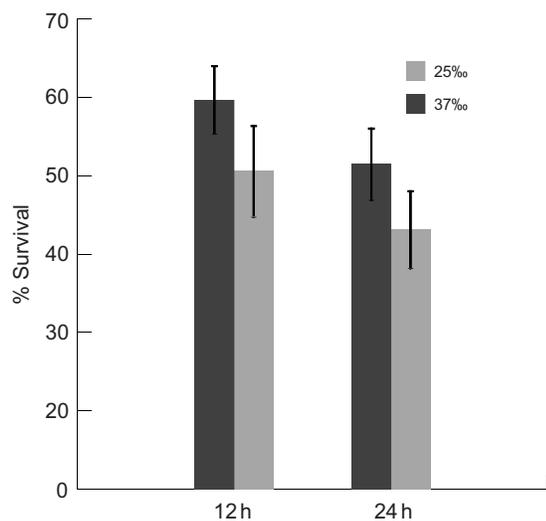

Fig. 2. Effect of the salinity that *G. gemineoa* larvae experience during development on their developmental success. Bars show the mean ± s.e.m. percentage of fertilised eggs that successfully develop into larvae after 12 and 24 h for nine experimental runs (replicates).
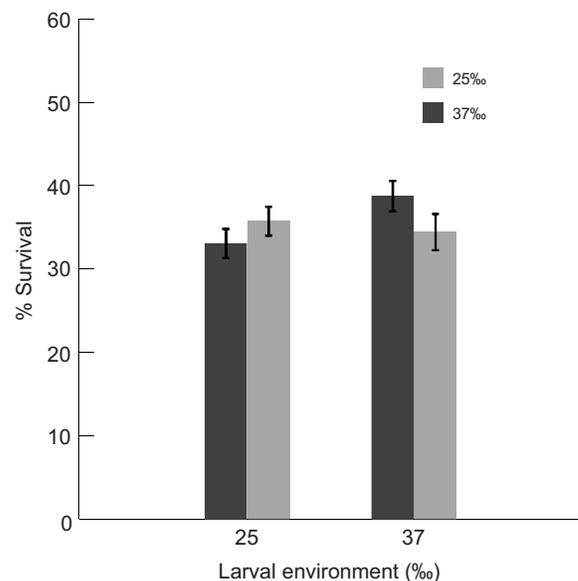

Fig. 3. Effect of the sperm and developmental salinity environment on the developmental success of *G. gemineoa* larvae. Bars show the mean ± s.e.m. percentage of fertilised eggs that successfully develop into larvae after 24 h for 16 experimental runs (replicates).

best when neither they nor their parental sperm were exposed to a lower salinity, whereas larvae survived worst when they were exposed to normal salinity but their parental sperm were exposed to low salinity. In other words, when sperm and larvae experienced the same environment, survival was generally higher than when the sperm and larval environments differed. These effects were similar regardless of the time that broodstock were collected and regardless of the site from which the broodstock were collected. This matching effect between the sperm environment and larval environment may represent an adaptive benefit but only if the sperm environment and larval environment are likely to covary in space and time (Marshall et al., 2010), otherwise this effect will only have negative consequences. Our results suggest that the sperm environment can be a significant source of variation in the subsequent phenotype of larvae. Previous studies have highlighted that metamorphosis does not reset all phenotypes to a common state (Pechenik, 2006); our results suggest that fertilisation similarly does not create phenotypically homogeneous zygotes, rather the environment that gametes experience can transcend fertilisation and affect subsequent performance.

The mechanism that drove our effects is unclear but can be divided into two (non-mutually exclusive) explanations: selection for specific sperm phenotypes and phenotypic plasticity. Sperm from the same ejaculate can vary in phenotype as a result of recombination during meiosis and developmental errors during spermatogenesis (Hunter and Birkhead, 2002). If sperm within an ejaculate vary in their resistance to lower salinity, then our experiments will have selected for a specific subset of sperm. Under this scenario, there must be some genetic link between the salinity tolerance of sperm and the viability of embryos, and this selection of specific sperm phenotypes results in a non-random distribution of embryo phenotypes. Interestingly, our results suggest that when the experience of offspring and sperm were matched, performance was greater overall – if sperm and larval tolerance to low salinity are indeed correlated, then selection on sperm salinity tolerance should increase larval tolerance to the same stress as was observed here. Links between sperm phenotypes and offspring phenotypes are often discussed in the sexual selection literature (Birkhead and Pizzari, 2002; Evans et al., 2003; Konior et al., 2009), but have received less attention from life-history theory. Nevertheless, evidence for covariance between sperm and offspring phenotypes is accumulating across a range of taxa (Droge-Young et al., 2012; Evans et al., 2007; Slatyer et al., 2012). Such covariance would result in the same types of constraints as discussed when phenotypic links exist between other life-history stages (Marshall and Morgan, 2011; Schluter et al., 1991). Viewed in this light, the phenotype of a sperm is the product of the selection pressures acting not only on the sperm but also on other life-history stages. Similarly, phenotypes in life-history stages may, to a certain degree, be a product of the selection pressures acting on sperm (Arnold, 1992).

Alternatively, our effects may have been driven by phenotypic plasticity. The exposure of sperm to lower salinity may have induced the differential expression of proteins across all sperm in that environment and these phenotypic changes in the sperm carried through to affect the phenotype and performance of offspring. The general view is that sperm do not possess the cellular machinery for translation and transcription, which is required for phenotypic plasticity (Suarez and Pacey, 2006). Nevertheless, in mammals, fertilisation ability is influenced by sperm capacitation; the post-ejaculatory environment plays a role in the expression of proteins, which determine capacitation phenotypes (Gur and Breitbart, 2006; Visconti et al., 1997; Zigler et al., 2005). Sperm may be translating nuclear encoded mRNA into important proteins coding for capacitation, which correlate with sperm motility and fertilising ability, suggesting they do have some ability to alter their phenotype after release according to the external environment (Gur and Breitbart, 2006; Visconti et al., 1997). Non-mammalian sperm are less well studied in this regard (but see Gasparini et al., 2012; Simmons et al., 2009), and we therefore cannot rule out either selection on sperm phenotypes or phenotypic plasticity as the mechanism driving the patterns we observed. We suspect, however, that the effects we observed were driven by phenotypic modification of the sperm – our treatments did not reduce fertilisation success, suggesting that sperm were not killed by our lower salinity, making selection effects less likely in our view.

Ecologically, our results show that highly transient environmental variation during the pre-fertilisation stage can manifest as differences in performance in the larval stage. It therefore appears that every life-history stage is linked phenotypically, representing a serious challenge to those seeking to understand the population dynamics of organisms with complex life cycles. For example, our results suggest that stresses could have more insidious and pervasive impacts than previously acknowledged. The gamete phase has long been recognised as the most sensitive to stress (Dinnel et al., 1989; Marshall, 2006). Our study suggests that exposure to a stress during fertilisation not only will affect the number of zygotes that are produced but also may alter the performance of those zygotes that are produced – a doubly negative effect of stress that could reverberate across the life history. These effects will occur even when the larval phase itself remains free from exposure to that stress. Interestingly, similar effects are observed when stress affects both larval survival and subsequent adult performance (Bergeron et al., 2011; Ng and Keough, 2003) and may be an important but unexplored source of variation in the performance of the adult stages of organisms with complex life cycles. Overall, our results suggest that the conditions in which organisms breed can have far-reaching effects on the phenotypes of their offspring. Here, we explored a relatively weak stress (only mildly reduced salinity); it would be interesting to explore the effects of stronger stresses such as very low salinity or anthropogenic stresses such as heavy metal exposure in future studies.

Our study was limited in a number of important ways. First, we only explored post-fertilisation performance for the 24 h after fertilisation. This was because we assumed that any effects of the sperm environment would manifest sooner rather than later. An important next step will be to explore effects throughout the life history. Second, we only explored the effects of varying the sperm environment for individual ejaculates – if selection drove the effects we observed, then we would expect stronger effects when we exposed multiple ejaculates to decreased salinity as this would provide more variation on which selection could act (Blows, 2007). As such, our results may represent an underestimate of the effects of the sperm environment on larval performance at the level of populations. Alternatively, if phenotypic plasticity drove our results, they may represent the upper limit of the effects of the sperm environment as our design minimised genetic sources of variation in larval performance by comparing offspring sired by the same father. Future studies will investigate whether the effects we observed are indeed stronger when multiple ejaculates are pooled. Finally, our study examined the effects of lower salinity on sperm alone; it would be very interesting to determine whether similar effects occur when eggs are exposed to environmental variation before fertilisation.

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AUTHOR CONTRIBUTIONS

D.J.M. and H.R. designed the experiments, H.R. executed the experiments, D.J.M. and H.R. analysed the data and D.J.M. and H.R. wrote the paper.

COMPETING INTERESTS

No competing interests declared.

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