

Population density effects on gamete traits and fertilisation dynamics under varying sperm environments in mussels

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October 31, 2023

Abstract

Gamete traits can vary widely among species, populations and individuals, influencing fertilisation dynamics and overall reproductive fitness. Sexual selection can play an important role in determining the evolution of gamete traits with local environmental conditions determining the strength and direction of sexual selection. Here we test for signatures of post-mating selection on gamete traits in relation to population density, and possible interactive effects of population density and sperm concentration on sperm motility and fertilisation rates among natural populations of mussels. Our study shows that males from high density populations produce smaller sperm compared with males from low density populations, but we detected no effect of population origin on egg size. Our results also reveal that females from low density populations tended to exhibit lower fertilisation rates across a range of sperm concentrations, although this became less important as sperm concentration increased. Variances in fertilisation success were higher for females than males and the effect of gamete compatibility between males and females increases as sperm concentrations increase. These results suggest that local population density can influence gamete traits and fertilisation dynamics but also highlight the importance of phenotypic plasticity in governing sperm-egg interactions in a highly dynamic selective environment.

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Running title: Gamete traits and fertilisation dynamics

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Here we test for signatures of post-mating selection on gamete traits in relation to population density, and possible interactive effects of population density and sperm concentration on sperm motility and fertilisation rates among natural populations of mussels. Our study shows that males from high density populations produce smaller sperm compared with males from low density populations, but we detected no effect of population origin on egg size. Our results also reveal that females from low density populations tended to exhibit lower fertilisation rates across a range of sperm concentrations, although this became less important as sperm concentration increased. Variances in fertilisation success were higher for females than males and the effect of gamete compatibility between males and females increases as sperm concentrations increase. These results suggest that local population density can influence gamete traits and fertilisation dynamics but also highlight the importance of phenotypic plasticity in governing sperm-egg interactions in a highly dynamic selective environment.

KEY WORDS : sexual selection, sperm competition, polyspermy, maternal effect, genotype by environment, cryptic female choice.

It is now widely recognised that in most sexually reproducing taxa sexual selection can continue after mating in the form of sperm competition, where ejaculates from rival males compete for fertilisation (Parker 1970), and cryptic female choice, where females influence the outcome of these contests (Eberhard 1996; Birkhead and Moller 1998; Parker 2020). This has resulted in a myriad of adaptations in both sexes; for example, selection can favour specific gamete traits that make ejaculates more competitive in the race to fertilise eggs (Pizzari and Parker 2009; Simmons and Fitzpatrick 2012; Lüpold et al. 2020), gamete plasticity in response to local environmental conditions (Crean and Marshall 2008), sperm chemoattractants that function to attract sperm from specific (e.g. compatible) males (Evans et al. 2012; Oliver and Evans 2014; Kekäläinen and Evans 2017; Lymbery et al. 2017), or gamete recognition proteins that ultimately determine whether sperm can fuse with an egg (Palumbi 1999; Swanson and Vacquier 2002; Levitan and Ferrell 2006; Evans and Sherman 2013).

Post-mating sexual selection has been argued to be particularly important in broadcast spawning organisms that release their gametes into the water for external fertilisation (reviewed in Evans and Sherman 2013; Kekäläinen and Evans 2018; Evans and Lymbery 2020). Indeed, one of the only opportunities for mate choice and mating competition in broadcast spawning organisms occurs through gamete-level interactions. However, broadcast spawning marine invertebrates face unique challenges that are linked to the spawning environment. In particular, variation in adult population density, and therefore the density of gametes in the water, is predicted to influence the intensity and direction of post-mating sexual selection (Parker et al. 1997; Franke et al. 2002; Levitan 2004; Levitan and Ferrell 2006; Marshall and Bolton 2007; Sherman et al. 2015; Hadlow et al. 2022). In low density populations, the rapid dilution of gametes can result in low fertilisation rates (Vogel et al. 1982; Levitan and Petersen 1995; Styán and Butler 2000; Levitan 2004), thereby generating selection on gamete traits in both sexes to increase the probability of egg-sperm encounters and fusion. For example, in females this may include the production of fewer, larger eggs to increase egg-sperm collision rates, greater investment in sperm-attracting chemoattractants, greater egg longevity, and less selective membrane blocks to sperm penetration (Yund 2000; Riffell et al. 2004; Marshall and Evans 2005; Levitan 2006; Levitan and Ferrell 2006; Kosman and Levitan 2014; Evans and Lymbery 2020). Under these sperm-limiting conditions males will maximise their fitness by increasing gamete encounter rates, for example by producing a smaller number of larger and/or longer lived sperm or altering swimming patterns (Parker 1993; Benzie and Dixon 1994; Parker 1998; Yund 2000; Snook 2005) (but see Crean and Marshall 2008).

At the other end of the population density spectrum, males from high-density populations face increased sperm competition, while females face an increased risk of polyspermy - where eggs are fertilized by two or more sperm (usually resulting in cell or zygote death). As a consequence, selection may favour female reproductive strategies that minimise the risk of polyspermy. Specifically, when sperm are abundant and the risks of polyspermy are high, females are predicted to produce smaller eggs, invest less in sperm-attracting chemoattractants, or increase the selectivity of membrane gamete recognition systems to reduce the risk of lethal polyspermy (Levitan and Ferrell 2006; Levitan et al. 2007; Kamel et al. 2010; Kosman and Levitan

2014). Males, on the other hand should produce higher numbers of smaller sperm (Parker 1998; Snook 2005; Parker 2006) or release smaller quantities of sperm over a longer period of time (Bode and Marshall 2007; Marshall and Bolton 2007). Consequently, as population density (and sperm abundance) increases, selection should favour reproductive strategies in males that further exacerbate the reproductive costs incurred by females (polyspermy) (Evans and Lymbery 2020). This complex interplay between population density and the differential selection pressures faced by males and females can lead to sexual conflict, where the optimal mating strategy of one sex reduces the reproductive fitness of the other (Parker 2006; Kamel et al. 2010). Despite this prediction, however, there are only a limited number of studies that have explored selection on gamete traits and fertilization dynamics from natural populations with varying adult densities (however, see Levitan 2002, 2004; Levitan and Ferrell 2006; Levitan 2012).

Blue mussels of the genus *Mytilus* are external spawners and provide an ideal experimental system for understanding the combined effects of population density, gamete traits and sperm abundance on fertilisation dynamics (Evans et al. 2012; Sherman et al. 2015; Evans and Lymbery 2020). Blue mussels are found on hard substrates of nearshore and intertidal habitats in temperate and subarctic regions of the northern and southern hemispheres (Hilbish et al. 2000) and are predominant species found in temperate Australian waters (Westfall and Gardner 2013; Ab Rahim et al. 2016; Popovic et al. 2020; Zbawicka et al. 2021). Populations vary naturally in population abundance (Cockrell et al. 2015) with likely concomitant variation in sperm concentrations among natural spawning events. Moreover, recent work on Australian *Mytilus* has revealed that ecologically relevant variation in sperm concentrations can have dramatic effects on fertilisation kinetics (Sherman et al. 2015). Furthermore, there is evidence from *Mytilus* for strong patterns of gamete selectivity, such that specific combinations of gametes from both sexes generate predictable differences in fertilisation rates and offspring viability (Evans et al. 2012; Oliver and Evans 2014; Sherman et al. 2015; Lymbery et al. 2017). However, the extent to which such patterns depend on the fertilisation environment has yet to be determined.

In this study we explore the effect of adult population density on variation in gamete traits and fertilisation dynamics across a range of sperm concentration environments in mussels from natural populations. We expected that selection will have acted on gamete traits in both sexes to optimise fertilization success under different egg-sperm ratios. Specifically, we explore whether population origin influences gamete traits in both sexes, and whether experimental adjustments to sperm-egg ratios during *in vitro* fertilisation trials influence fertilisation kinetics and pairwise patterns of fertilisation rates (i.e. gamete compatibility). One expectation from the latter trials is that eggs will become more selective as the risk of polyspermy (high sperm concentrations) increase, resulting stronger gametic (male-by-female) interactions under high sperm densities.

Materials and Methods

COLLECTIONS AND SPAWNING OF ANIMALS

We collected mussel broodstock from high and low density sites within each of three locations in Port Phillip Bay, Victoria, Australia during the May winter spawning season. These included Williamstown (-37.861695°, 144.912751°), Geelong (-38.148770°, 144.390864°) and Portarlington (-38.111790°, 144.659556°). Mussels were transported to the Victorian Marine Science Consortium research laboratories at Queenscliff and held in flow-through tanks at ambient temperature (16 °C). All animals were cleaned of epiphytes and other debris and used for spawning on the day of collection using the approach of Pettersen et al. (2010).

COLLECTION OF GAMETES

Gametes were harvested by inducing spawning using standard thermal stress protocols (Pettersen et al. 2010). This involved cycling seawater temperature between 16-24 °C to induce spawning. Males and females were identified at the time of gamete release, rinsed with filtered seawater, and isolated into individual spawning chambers (120 × 175 × 70 mm). Eggs were rinsed through a 125µm mesh, and sperm through a 30µm mesh, to remove any debris released from the adult mussel during spawning. The concentration of sperm for each male was determined from three replicate counts using an improved Neubauer haemocytometer and sperm

concentrations were initially standardized to 2×10^8 sperm ml^{-1} . A serial sperm dilution was then carried out to obtain the desired experimental concentrations of sperm for our experiments: 2×10^2 ; 2×10^3 ; 2×10^4 ; 2×10^5 ; 2×10^6 ; and 2×10^8 sperm ml^{-1} . Egg concentrations were estimated from three replicate counts using a Beckman multisizer 3 Coulter counter and standardized to 2000 eggs ml^{-1} (stock egg solution).

FERTILISATION ASSAYS

Our block cross-classified design involved a series of 2x2 *in vitro* fertilisation trials (following the analogous block North Carolina II quantitative genetic breeding design described by Lynch and Walsh (1998)), each involving two males (one from a high density and one from a low density site from the same location) crossed with two females (one from a high and one from a low density site from the same location) in every pairwise combination. For each male-female cross, two replicate fertilisation assays were carried out across each of the six different sperm concentrations, giving a total of 48 fertilisation assays per block. A total of five blocks were carried out for each of the three locations sampled, giving a total of 15 blocks and 720 fertilisation assays across all three locations. We conducted fertilisation assays in sterile 24-well cell culture plates with a total fertilisation volume of 2 ml. This consisted of 1 ml of the stock egg solution (2000 eggs total), and 1 ml of sperm solution, resulting in a final egg concentration of 1000 eggs ml^{-1} and sperm concentrations of: 1×10^2 ; 1×10^3 ; 1×10^4 ; 1×10^5 ; 1×10^6 ; and 1×10^8 sperm ml^{-1} . All fertilisation assays within a block were conducted immediately after gamete standardisation and within 1 minute of each other although we also ensured that the time from the start of spawning to fertilisation was recorded and included in the analysis to control for potential gamete aging effects ('gamete age' in the model). Fertilisation assays were left at room temperature for 3 hours before samples were fixed with 1% formalin. For each fertilisation assay, a random sub-sample of approximately 100 eggs were observed under an inverted microscope at 400x magnification and the number of fertilised eggs were distinguished from unfertilised eggs by counting the number of cells that had undergone cell division, or had a clearly visible fertilisation envelope. Unfertilised eggs were identified from the lack of cell division or the absence of a fertilisation envelope (Sherman et al 2015). Abnormal eggs were identified by signs of irregular cleavage, incomplete blastula development and by dissolution of the egg membrane resulting in deformed embryos and/or the breaking apart of the developing cell cluster (Lewis and Galloway 2009).

GAMETE TRAITS AND MEASURES OF ADULT BODY CONDITION

Female egg size was assessed from three replicate counts using a Beckman multisizer 3 Coulter counter (mean number of eggs per female = 8,371 $\pm$ 1140 SE). To estimate sperm size, digital photographs of sperm were taken using a digital camera attached to a microscope (OLYMPUS IX53, 20X objective). Sperm length was measured for each male using the software ImageJ (<http://rsb.info.nih.gov/ij>). Sperm length was measured in μm as the distance from the tip of the head (excluding the protruding acrosomal cap or 'beak') to the end of the tail. Ten sperm were measured for each male, and the average of the 10 measures was used in the analysis.

Sperm motility was assessed using computer-assisted sperm analysis (CEROS sperm tracker, Hamilton-Thorne, Beverly USA). Sperm motility was assessed at two sperm concentrations (10^6 and 10^8). For each individual, two 4 μl aliquots of the sperm solution were placed in two separate wells of a 12-well Multi-test slide and then covered with a coverslip. Sperm motility was therefore assessed in two subsamples for each male at both sperm concentrations. Given the high reported repeatability of sperm motility measures in blue mussels (Fitzpatrick et al. 2012), we recorded the average of the two subsamples for the ensuing analysis, and caution was taken to analyse sperm in a random order with respect to the sperm concentration used. From the CASA analyses, we obtained the percentage of motile sperm and a series of parameters describing velocity and trajectory of motile sperm. These parameters include average path velocity (VAP), straight-line velocity (VSL), curvilinear velocity (VCL), lateral head displacement (ALH), beat cross frequency (BCF), straightness (STR) and linearity (LIN). Cut offs for static cells were the same as those used in previous experiments by Eads et al. (2016). An average of 276.8 ± 37.0 SE sperm tracks were analysed for each male at each concentration.

As body condition may influence fertilisation success (i.e. mussels in better condition may have higher quality gametes) we controlled for this potential confounding effect in our analysis. We recorded shell length (mm) and flesh mass (g) from each brood parent and used these to calculate a condition index using the residuals of flesh weight against shell length.

STATISTICAL ANALYSIS

Differences in egg size between high- and low-density sites were assessed using a General Linear Mixed Model (GLMM). This included egg size as the response variable, density as a predictor (fixed effect), location (random effect) and female condition as a covariate. Similarly, differences in sperm length between low- and high-density sites were assessed using sperm length as the response variable, density as a predictor (fixed effect), location (random effect) and male condition as a covariate.

Principal component analysis (PCA) was used to reduce the motility parameters to two PCs with eigenvalues more than 1, explaining respectively 60.75% and 23.35% of the variance (total variance explained: 84.10%). PC1 was predominantly loaded by the three velocity measures (VAP, VSL & VCL) and is therefore interpreted as a measure of sperm swimming speed; PC2 was loaded most strongly by STR and LIN and is therefore interpreted as a measure of sperm swimming straightness. These two PCs (PC1 and PC2) were used in all subsequent analyses, and their relationship with the original motility parameters is detailed in Table 1. We analysed differences in sperm motility between low- and high-density populations at the two sperm concentrations (10^6 and 10^8 , see above) using a General Linear Mixed Model (GLMM) within the lme4 package of R using PC scores (PC1 and PC2) as dependent variables, population density and sperm density as fixed factors with their interactions, location as a random effect and male condition as a covariate. The same model was run to analyse the percentage of motile sperm (arcsine-transformed before use). Means are presented with their standard errors (SE).

We analysed fertilisation success using Markov-chain Monte-Carlo generalized linear mixed models in R (package MCMCglmm, (Hadfield 2010)). With this Bayesian mixed model approach, we modelled the proportion of normally and abnormally fertilised eggs following a binomial distribution and obtained both an estimate of the components of variance and an estimate of the interval of credibility. All models were run for 1.3×10^7 iterations, with a thinning interval of 100 (i.e., only one iteration from every 100 in the Markov chain was used to estimate the posterior distribution of the parameters to reduce the occurrence of autocorrelation between successive iterations), and a burn-in of 3×10^5 (i.e., we discarded the first 3×10^5 models of the simulation to avoid issues with autocorrelation).

For the Bayesian models we included the fixed effects of population (3 levels, Geelong, Portarlington, and Williamstown), gamete age (time elapsed between spawning and fertilisation), body condition (see above), sperm concentration (fitted both as a linear and quadratic term), density of the male (high vs low), density of the female (high vs low), their interaction with each other and with sperm concentration. The random effects used in the model included block (V_{block}), identity of male (V_{male}), identity of female (V_{female}), and an interaction between male and female IDs ($V_{\text{male} \times \text{female}}$). The residual variance (V_e) of the model represents the variance between replicates within a given male and female pair.

A necessary step in Bayesian statistical analyses is to set priors before running the models. The term prior refers to the prior distribution of a parameter before the data are analysed. The level of information of the prior can vary from noninformative to highly informative. When knowledge about the relationship between the variables in the model is low, it is best to run the model with different priors and to check whether these different priors provide different posterior distributions (Hadfield 2010). We therefore ran the models using inverse Wishart priors (equivalent to an inverse gamma distribution with shape=scale=0.001; $V=1$, $\nu=0.002$), parameter expanded priors ($V=1$, $\nu=1$, $\alpha.\mu=0$, $\alpha.V=1000$), and non-informative improper priors ($V=1e-16$, $\nu=-2$). Although we present results from the model using parameter expanded priors, the conclusions did not qualitatively change according to prior specifications. We inspected the 95% highest posterior density (HPD) intervals associated with each fixed effect to check whether they overlapped with zero. A 95% HPD interval contains most of the posterior distribution and is analogous to a confidence

interval in the frequentist approach; we considered estimates with 95% HPD intervals overlapping with zero as non-significant.

We ran an initial model for the proportion of normally fertilised eggs while excluding data from the highest sperm concentration to facilitate the interaction between sperm concentration and population density (i.e., including the highest sperm concentration made the fertilisation curve very complex). To model the proportion of abnormally fertilised eggs, we ran a second model while keeping data in the highest sperm concentration. The proportion of abnormally fertilised eggs was very low at other sperm concentrations.

We ran a third model, this time including data from all sperm concentrations and allowed variance components to vary with sperm concentration. We used the “`idh()`” function to model heterogeneous variance components for V_{male} , V_{female} , and $V_{\text{male} \times \text{female}}$ according to each concentration. Hence, there were 20 variance components estimated in this model (i.e., V_{male} , V_{female} , and $V_{\text{male} \times \text{female}}$ estimated separately for sperm concentrations and a single V_{block} and V_e). By contrast to Gaussian data, with binomial data it is not recommended to compare different models using the deviance information criteria (an index produced by MCMCglmm models that balances the fit of the model based on the number of parameters used in the model). Thus, we cannot formally test whether model fit was improved by allowing heterogeneous variance components across sperm concentrations. Instead, we inspected the 95% HPD intervals associated with each random effect to check whether they overlapped. We considered estimates with non-overlapping 95% HPD intervals as significantly different (Hadfield 2010). Note that, as the lower limit of a variance component is bound to zero, its lower 95% HPD can be extremely close to, but cannot overlap zero. Thus, inspection of the HPDs cannot be formally used to test whether a variance component is significantly greater than zero (Hadfield 2010). Nevertheless, the 95% credible intervals around the variance estimates provide a measure of the precision of the estimate and allowed us to test whether $V_{\text{male} \times \text{female}}$ differed across sperm concentrations.

Data Availability statement

Data and associated r-code for the fertilisation analysis can be found here: <https://datadryad.org/stash/share/Yx-wxEJsD-SjrHkHG9lVShv1Qw-KFTAT55vBMvUBVQ4>

RESULTS

GAMETE TRAITS

Egg size did not differ significantly between females from high and low density sites (mean egg size: Female_(high) = 64.36 $\mu\text{m} \pm 0.359$ versus Female_(low) = 64.35 $\mu\text{m} \pm 0.244$, GLMM: $F_{1, 29} = 0.01$, $P = 0.939$). However, we did detect a significant difference in egg size among locations (mean egg size: Williamstown = 64.27 μm ; ± 0.233 Geelong = 65.65 $\mu\text{m} \pm 0.179$; Portarlington = 63.43 $\mu\text{m} \pm 0.308$, GLMM: $F_{2, 29} = 18.39$, $P < 0.0001$). Our analysis of sperm length revealed a significant difference between high- and low-density sites (GLMM: $F_{1, 29} = 5.01$, $P = 0.035$), with sperm from low density sites being longer compared with sperm from high density sites (mean sperm length: low density = 51.13 $\mu\text{m} \pm 0.547$ cf. high density = 49.86 $\mu\text{m} \pm 0.387$). There was no significant difference in sperm length among locations (GLMM: $F_{2, 29} = 0.11$, $P = 0.893$).

There was no effect of population density ($F_{1, 24.159} = 0.4036$, $P = 0.531$) or sperm density ($F_{1, 25.2} = 0.520$, $P = 0.478$) on sperm motility PC1 scores, or their interaction ($F_{1, 25.1} = 0.04$, $P = 0.845$). Similarly, PC2 scores were not affected by population density ($F_{1, 28.5} = 0.25$, $P = 0.620$), sperm density ($F_{1, 29.8} = 0.24$, $P = 0.627$) or their interactions ($F_{1, 29.6} = 0.07$, $P = 0.797$). Male condition also did not affect sperm motility PC1 ($F_{1, 37.6} = 0.13$, $P = 0.909$) or PC2 ($F_{1, 43.9} = 0.63$, $P = 0.431$) scores. The percentage of motile sperm was not affected by population density ($F_{1, 27.6} = 0.04$, $P = 0.841$), but it was affected by sperm density ($F_{1, 27.1} = 0.45$, $P < 0.001$). We also detected no significant effects of the interaction between population and sperm density ($F_{1, 26.9} = 0.014$, $P = 0.907$) or the effect of male condition ($F_{1, 37.1} = 1.80$, $P = 0.882$) on the percentage of motile sperm. Sperm were on average more motile at the 1×10^8 concentration (31.82% ± 2.22) compared to the 1×10^6 concentration (14.42% ± 2.85).

FERTILISATION

The percentage of successfully fertilised eggs steeply increased with sperm concentration but dropped significantly beyond 1×10^6 (Fig. 1a). Beyond this concentration, ~60 to 80% of eggs were abnormally fertilised (Fig. 1b). Therefore, had we simply counted fertilised vs unfertilised eggs, without making a distinction between normally vs abnormally fertilised eggs, we would have seen a continuous increase in proportion of fertilised eggs (Fig. 1c) while in fact many of the fertilised eggs at the highest sperm concentration (1×10^8) were non-viable.

In the first Bayesian model, we analysed the proportion of normally fertilised eggs after excluding the highest sperm concentration (1×10^8). There was a significant effect of population of origin, sperm concentration (both linear and quadratic terms), female density, and a significant interaction between female density and sperm concentration (Table 2). Females from low density areas had lower fertilisation success across all sperm concentrations (Fig. 1a), but the significant interaction indicated that this effect becomes less important as sperm concentration increases (Table 2). We detected no effect of male density on fertilisation success (Table 2). In the second model, in which we analysed the proportion of abnormally fertilised eggs at the highest sperm concentration (1×10^8), we found no significant effect of population, body condition, gamete age, female density, male density (or their interaction) on the proportion of abnormally fertilised eggs (Table 3).

Finally, our third Bayesian model revealed some differences in the variance estimates across sperm concentrations. The variance estimates associated with intrinsic male effects (V_{male}) were low across all sperm concentrations (range: <0.01 to 0.01 ; Table 4). By contrast, effects attributable to females (V_{female}) were relatively high (range: 0.62 to 4.96 ; Table 4). The “male $\times$ female” interaction effects ($V_{\text{male} \times \text{female}}$) were relatively low at low sperm concentration (Fig. 2), but increased at higher sperm concentration (Table 4, Fig. 2).

Discussion

In this study we tested for signatures of post-mating selection on gamete traits in relation to population density, and possible interactive effects of population density and sperm concentration on sperm motility and fertilisation rates. Our study yielded four key results: 1) males from high density populations produce smaller sperm compared with males from low density populations, although we found no difference in egg size between females from different population densities; 2) females from low density populations have lower fertilisation success, although this becomes less important as sperm concentration increases; 3) variances in fertilisation success were higher for females than males; and 4) gamete compatibility between males and females increases as sperm concentrations increase.

We found that males from high density populations, where sperm competition would be expected to be more prevalent (Evans and Lymbery 2020), produced smaller sperm compared to males from low density populations. To the extent that sperm production trades-off against sperm size (Parker 1982; Gomendio et al. 1991; Tourmente et al. 2011), this result supports the prediction that an increase in the overall magnitude of sperm competition should select for a greater number of smaller sperm, while lower sperm competition risk should result in the production of fewer longer sperm (Parker 1982; Stockley et al. 1997; Gage and Morrow 2003; García-González and Simmons 2007; Lüpold et al. 2020). These results support comparative studies of fish that show sperm length decreased with sperm competition risk (Stockley et al. 1997), although some studies have found the opposite, with among species comparisons showing a positive relationship between sperm competition risk and sperm size (Gage 1994; Byrne et al. 2003; Lifjeld et al. 2010; Johnson et al. 2013). The reason for such varying and contrasting results likely arises from the complex relationship between sperm morphology and sperm performance (e.g. swimming speed and/or fertilisation potential). In some species (especially internally fertilisation taxa) longer sperm may be associated with improved swimming performance and provide a competitive advantage under sperm competition (Gomendio et al. 1991; Parker et al. 2010; Johnson et al. 2013). However, sperm length may also be associated with sperm longevity, with longer, larger sperm living longer than smaller sperm. If so, producing longer-lived sperm may confer an important advantage in broadcast spawners in low density populations, allowing more time for sperm-egg encounters and increased fertilisation success (however see, Levitan 2000). A study of the broadcast spawning tubeworm *Galeolaria gemineoa* showed that sperm with small heads but long tails were favoured in high-

concentration environments, whereas sperm with long heads were favoured at low concentrations and old ages (Johnson et al. 2013). The longer sperm produced by males from low density populations detected in this study may provide an advantage if they have greater longevity, although longevity was not directly assessed in this study. Our analysis also showed that longer sperm from low density populations did not show greater motility or swimming speeds, suggesting that the larger size did not confer a swimming advantage. The relation between sperm size and fertilisation success under sperm competition is complex and appears to be highly context dependent and likely to covary with sperm swimming traits and longevity.

Theoretical and empirical studies predict that egg size should reflect adult density, with females from low-density populations producing larger eggs than their high-density counterparts, because larger eggs present larger targets for searching sperm (Levitan 2006; Crean and Marshall 2008; Evans and Lymbery 2020). In the present study we found no difference in mean egg size between females from high- and low-density populations. However, egg size is not the only mechanisms available to females for increasing the effective target size of their eggs. For example, it is well established that the eggs of many broadcast spawners release sperm chemoattractants, which are thought to increase the effective target size of eggs, thus making them more ‘visible’ to searching sperm (e.g. for theory see Jantzen 2001). While we currently lack explicit evidence that females can facultatively adjust the production of sperm chemoattractants to match the fertilisation environment, or that selection may favour increased production of such attractants in populations with persistently low adult densities (and therefore sperm limited), the idea of testing for differences either in the composition or volume of sperm chemoattractants across populations has merit. Such an adaptation may represent a more cost-effective mechanism for increasing the target size of eggs compared to increasing the structural size of eggs in sperm-limited environments and offers an exciting area for future research.

We assessed fertilisation rates of females from low- and high-density populations across a range of sperm concentrations. As population densities decrease and the risk of sperm limitation increases, we expected that females would produce eggs that are more readily fertilised (i.e. greater fertilisation success at lower sperm concentrations), while in high sperm density environments females should increase ovum defences to reduce the risk of polyspermy (Frank 2000; Firman and Simmons 2013; Kosman and Levitan 2014). Surprisingly, we found no evidence to support these ideas. Indeed, we found that females from high density sites produced eggs that were more readily fertilised at lower sperm concentrations than eggs from females originating from low density populations, although this difference became progressively less apparent as sperm concentrations increased. Furthermore, our finding that at the highest sperm concentration (2×10^8 sperm ml^{-1}), females from both high- and low-density populations experienced similar magnitudes of abnormally fertilised eggs fails to support the idea that females from high density populations are better at blocking polyspermy compared to females from low density populations (Kosman and Levitan 2014).

Interestingly, we found that the variance components for (normal) fertilization rates across the range of sperm concentrations were generally higher in females than in males, but also that variances for females were initially high at lower sperm concentrations (2×10^2 & $2 \times 10^3 \text{ml}^{-1}$), decreased when sperm concentrations were at an intermediate level (2×10^4 & $2 \times 10^5 \text{ml}^{-1}$), before rising sharply when sperm concentrations exceeded $2 \times 10^6 \text{ml}^{-1}$. Qualitatively similar patterns have been reported in other broadcast spawning invertebrates (Levitan 2004), suggesting that under sperm limitation, where average fertilisation rates are low, there will be heightened opportunity for selection on female traits that improve fertilisation rates. Under such conditions, for example, we might expect stronger selection for increased egg size and/or the heightened production of chemoattractants that increase sperm-egg encounter rates (see Evans and Lymbery 2020). Under intermediate sperm concentrations, fertilisation rates were generally high ($\sim 80\%$) and the variance in normal fertilisation rates was consequently very low. By contrast, despite high overall fertilisation rates in the highest sperm concentration groups ($> 2 \times 10^6 \text{ml}^{-1}$), the proportion of normally fertilised eggs decreased dramatically at high sperm concentrations with a concomitant increase in the variance in normally fertilised eggs (see Fig. 1a). Together these findings suggest that the opportunity for selection on egg traits will depend critically on local spawning conditions, and that such patterns may be reflected on a broader spatial scale in divergent populations with varying adult densities.

Our fertilisation assays indicated that as sperm concentration increased, gametic compatibility effects became increasingly important. This suggests that under sperm limited conditions, where the risk of fertilisation failure is higher, any benefits associated with selecting genetically compatible sperm (Kosman and Levitan 2014; Oliver and Evans 2014) are offset by the direct costs of leaving many eggs unfertilised. However, as sperm concentration increases, eggs can afford to become ‘choosier’ as this will ensure that fertilisations are biased toward genetically compatible sperm (Sherman et al. 2015; Lymbery et al. 2017) whilst avoiding the direct costs associated with polyspermy. Although we have yet to understand the mechanistic basis that underlie these dynamics patterns of sperm-egg interaction, we suspect that sperm chemoattraction and gamete surface proteins play important roles in differentially regulating sperm-egg encounter rates across the sperm concentration continuum (Evans and Sherman 2013). Irrespective of the underlying mechanisms, our results provide further support to an increasing number of studies that have shown that local environmental conditions can influence the magnitude of compatibility effects between male-female combinations (Levitan and Ferrell 2006; Levitan et al. 2007; Nystrand et al. 2011; Lymbery and Evans 2013; Sherman et al. 2015; Rudin-Bitterli et al. 2018). Collectively, these studies highlight the importance of testing for compatibility effects across a range of ecologically relevant environmental conditions.

In conclusion, our study revealed significant effects of population density and sperm concentration on gamete morphology and fertilisation dynamics, respectively. While the extent of variation at the population level may be determined by both environmental and/or genetic factors, the critical importance of gamete-level natural and sexual selection in broadcast spawners (Evans and Sherman 2013; Evans and Lymbery 2020) leads us to predict that much of the variation we observe in these populations is adaptive. However, we also report highly dynamic patterns of fertilisation across experimentally altered sperm environments, highlighting the importance of phenotypic plasticity in governing sperm-egg interactions and the likely dynamic selective environment in which fertilisation plays out. We eagerly anticipate future work that seeks to understand the extent to which the mechanisms underlying these dynamic patterns of sperm-egg interaction are themselves plastic in their expression.

ACKNOWLEDGEMENTS

The authors would like to thank the Victoria Shellfish Hatchery for advice and support in the collection and spawning of mussels. Funding was provided by grants from the Australian Research Council (ARC Discovery) to JPE (DP150103266) and Deakin University to CDHS. We thank the Deakin Queenscliff Marine Science Centre for use of the aquarium and seawater systems.

Conflict of interest

The authors declare no conflict of interest.

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Table 1. Principal component analysis of sperm motility parameters. These parameters include average path velocity (VAP), straight-line velocity (VSL), curvilinear velocity (VCL), lateral head displacement (ALH), beat cross frequency (BCF), straightness (STR) and linearity (LIN). Correlation coefficient (r) and the percentage contribution of each parameter to the principal component score are provided.

Component	PC1	PC1	PC2	PC2
	r	%	r	%
VAP	0.94	20.78	-0.30	5.62
VSL	0.98	22.62	0.11	0.77
VCL	0.87	17.87	-0.38	8.89
ALH	0.89	18.44	-0.20	2.49
BCF	-0.49	5.69	0.26	4.07
STR	0.38	3.40	0.90	49.86
LIN	0.69	11.21	0.68	28.30

Table 2. Parameters from a mixed model of proportion of eggs normally fertilized across different sperm concentration in mussels, fitted using a Bayesian approach. Shown are posterior means and the 95% highest posterior density (HPD) intervals for (a) random effects of measurement block (V_{block}), male identity (V_{male}), female identity (V_{female}), specific combinations of males and females ($V_{\text{male} \times \text{female}}$), and specific environment (V_e ; residual variance) and (b) fixed effects of population (3 levels; Geelong as the reference), body condition, gamete age (minutes), sperm concentration (continuous variable fitted both as a linear and quadratic term), density of the male, density of the female, and their interactions with each other and with sperm concentration.

		95% HPD interval	95% HPD interval
	estimate	lower	upper

			95% HPD interval	95% HPD interval
(a) random effects:	(a) random effects:			
	V_{block}	0.706	0.000	2.223
	V_{male}	0.058	0.000	0.184
	V_{female}	1.416	0.575	2.500
	$V_{\text{male} \times \text{female}}$	0.045	0.000	0.127
	V_e	1.430	1.224	1.630
(b) fixed effects:	(b) fixed effects:			
	intercept	0.443	-2.076	3.276
	population[Portarlinton]	-0.702	-2.262	1.056
	population[Williamstown]	-1.411	-3.393	0.345
	male body condition index	-0.255	-0.508	-0.046
	female body condition index	-0.203	-0.894	0.637
	male gamete age	0.001	-0.008	0.010
	female gamete age	0.000	-0.011	0.011
	sperm concentration	33.126	28.735	37.249
	sperm concentration²	13.124	10.690	15.503
	male density _[low]	0.113	-0.442	0.671
	female density_[low]	-1.308	-2.266	-0.225
	male _[low] $\times$ female _[low] density	-0.106	-0.551	0.375
	male density _[high] $\times$ sperm concentration	-0.027	-0.178	0.113
	female density_[low] $\times$ sperm concentration	0.195	0.062	0.341

Table 3. Parameters from a mixed model of proportion of eggs abnormally fertilized at the highest sperm concentration in mussels, fitted using a Bayesian approach. Shown are posterior means and the 95% highest posterior density (HPD) intervals for (a) random effects of measurement block (V_{block}), male identity (V_{male}), female identity (V_{female}), specific combinations of males and females ($V_{\text{male} \times \text{female}}$), and specific environment (V_e ; residual variance) and (b) fixed effects of population (3 levels; Geelong as the reference), body condition, gamete age (minutes), density of the male, density of the female, and their interaction.

		Estimate	95% HPD interval Lower	95% HPD interval Upper	Probability (MC)
(a) random effects:	(a) random effects:				
	V_{block}	3.928	0.001	11.090	
	V_{male}	0.834	0.000	3.418	
	V_{female}	1.788	0.000	5.956	
	$V_{\text{male} \times \text{female}}$	4.100	1.136	7.083	
	V_e	0.113	0.031	0.203	
(b) fixed effects:	(b) fixed effects:				
	intercept	2.762	-3.245	7.958	0.282
	population[Portarlinton]	-1.504	-4.822	2.203	0.356
	population[Williamstown]	-3.039	-6.818	1.163	0.130
	male body condition index	0.218	-0.690	1.312	0.648
	female body condition index	0.460	-0.827	1.963	0.528
	male gamete age	0.015	-0.008	0.035	0.192
	female gamete age	-0.010	-0.029	0.011	0.324
	male density _[low]	-0.606	-2.470	1.123	0.510
	female density _[low]	0.725	-1.007	2.613	0.416
	male _[low] $\times$ female _[low] density	0.618	-1.378	2.896	0.548

Table 4. Variance estimates from a mixed model of proportion of eggs normally fertilized across different sperm concentration in mussels, fitted using a Bayesian approach. Shown are posterior modes and the 95% highest posterior density (HPD) intervals for random effects of measurement block (V_{block}), male identity (V_{male}), female identity (V_{female}), specific combinations of males and females ($V_{\text{male} \times \text{female}}$), and specific environment (V_e ; residual variance). V_{male} , V_{female} , and $V_{\text{male} \times \text{female}}$ were fitted heterogeneously for each sperm concentration.

variance component	posterior mode	95% HPD lower	95% HPD upper
V_{block}	0.28	<0.01	1.82
$V_{\text{male}} [2 \times 10^2]$	<0.01	<0.01	0.05
$V_{\text{male}} [2 \times 10^3]$	<0.01	<0.01	0.03
$V_{\text{male}} [2 \times 10^4]$	<0.01	<0.01	0.08
$V_{\text{male}} [2 \times 10^5]$	0.01	<0.01	2.19
$V_{\text{male}} [2 \times 10^6]$	0.01	0.00	1.25
$V_{\text{male}} [2 \times 10^8]$	0.02	0.00	2.71
$V_{\text{female}} [2 \times 10^2]$	1.51	0.68	2.68
$V_{\text{female}} [2 \times 10^3]$	1.12	0.56	2.18
$V_{\text{female}} [2 \times 10^4]$	0.62	0.23	1.49
$V_{\text{female}} [2 \times 10^5]$	1.96	0.00	4.97
$V_{\text{female}} [2 \times 10^6]$	4.67	1.28	16.19
$V_{\text{female}} [2 \times 10^8]$	4.96	1.16	41.24
$V_{\text{male} \times \text{female}} [2 \times 10^2]$	0.06	<0.01	0.19
$V_{\text{male} \times \text{female}} [2 \times 10^3]$	<0.01	<0.01	0.12
$V_{\text{male} \times \text{female}} [2 \times 10^4]$	0.18	0.09	0.42
$V_{\text{male} \times \text{female}} [2 \times 10^5]$	0.75	0.27	1.65
$V_{\text{male} \times \text{female}} [2 \times 10^6]$	0.56	0.11	1.17
$V_{\text{male} \times \text{female}} [2 \times 10^8]$	2.33	0.99	4.47
V_e	0.05	0.03	0.07

Figure 1. Proportion of (a) normally, (b) abnormally, and (c) total (=normally+abnormally) fertilized eggs as function of sperm concentration in mussels collected from high (H) and low (L) population density and mated in a fully factorial fertilisation design. For each experimental block, four females were paired with four males (and *vice versa*) at six different sperm concentrations. Each line within a panel represent the mean of a specific male and female population density. Hence, comparing the red vs blue lines shows the difference between H and L females. Comparing squared vs circles shows the differences between H and L males.

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Figure 2. Variance in fertilization success attributed to the interaction between male and female identity ($V_{\text{male} \times \text{female}}$) across sperm concentrations in mussels. Black dots show posterior modes and the lines show 95% confidence intervals (CI; highest posterior density intervals) from the MCMCglmm model.

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