

Effects of mating order and male size on embryo survival in a pipefish

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In species that provide parental care, individuals should invest adaptively in their offspring in relation to the pre- and post-zygotic care provided by their partners. In the broad-nosed pipefish, *Syngnathus typhle* L., females transfer large, nutrient-rich eggs into the male brood pouch during mating. The male broods and nourishes the embryos for several weeks before independent juveniles emerge at parturition. Given a choice, females clearly prefer large partners. Yet, females provide protein-richer eggs when the same individual mates with a smaller than a larger male. In the present study, we allowed each female to mate with one small and one large male, in alternated order. We found a strong effect of female mating order, with larger clutches and higher embryo mortality in first- than second-laid broods, which may suggest that eggs over-ripen in the ovaries or reflect the negative effects of high embryo density in the brood pouch. In either case, this effect should put constraints on the possibility of a female being selective in mate choice. We also found that small and large males produced embryos of similar size and survival, consistent with the reproductive compensation hypothesis, suggesting that, in this species, larger males provide better nourishment to the embryos than smaller males. © 2014 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2015, **114**, 639–645.

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INTRODUCTION

The relationship between mate quality and parental care is central to the evolution and maintenance of mate choice in sexually reproducing species (Trivers, 1972; Parker, 1983). Mate choice should occur when phenotypic variation between mates in a population reliably advertises mate quality (Zahavi, 1975), enabling individuals to gain fitness benefits in terms of offspring number, health or viability (Hoelzer, 1989;

Price, Schluter & Heckman, 1993; Gowaty, 2008). However, social and ecological constraints often prevent individuals from mating with preferred partners (Gowaty, 2008). When less-preferred mates are a better option than not mating at all, reproduction may result in offspring with viability deficits compared to those produced in reproductive events with preferred partners (Gowaty *et al.*, 2007; Gowaty, 2008). In such situations, individuals may compensate by increasing their reproductive effort to achieve an offspring fitness comparable to when mating with a partner of their choice; a strategy termed reproductive compensation (Gowaty *et al.*, 2007; Gowaty,

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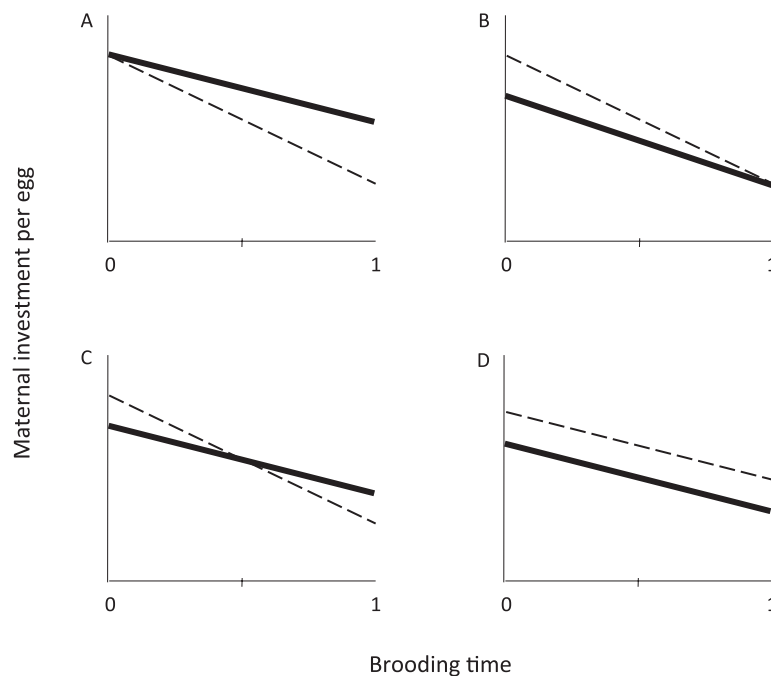


Figure 1. Patterns of zygote energy content during development in the presence or absence of reproductive compensation. Zygotes lose energy content as they develop into independent young as a result of metabolism, unless nutrients are added during the brooding period. In these graphs, time = 0 gives the maternal investment per egg at mating and time = 1 the offspring's energy content at independence. High (solid) or low (dashed) male quality care may cause the decline in energy content of a fertilized egg to be slow or rapid, respectively. Four patterns can be predicted depending on whether low quality care is (A) not, (B) fully or (C) partially compensated for by the female, by means of a higher investment into the unfertilized egg, or (D) whether quality of care does not differ but initial investment does. In this simple heuristic model, the decline is shown to be linear, although other relationships may also apply.

2008). As zygotes develop into independent young, their energy content typically declines unless nutrients are added during brooding (Jaworski & Kamler, 2002). For species in which the quality of care may cause this decline to be slow or rapid, three patterns can be predicted, depending on whether reproductive compensation is absent (Fig. 1A), complete (Fig. 1B) or partial (Fig. 1C).

The broad-nosed pipefish, *Syngnathus typhle*, is characterized by predominant male mate choice and female competition for mating opportunities (Berglund, Rosenqvist & Svensson, 1986b, 1989; Berglund, Widemo & Rosenqvist, 2005). Females produce large nutritious eggs continuously (i.e. egg maturation is asynchronous) (Sogabe & Ahnesjö, 2011). During mating, eggs are transferred to the male brood pouch. Males brood the offspring for several weeks, during which time they provide osmoregulation, oxygenation, and proteins (Berglund *et al.*, 1986b; Braga Goncalves, 2010; Kvarnemo *et al.*, 2011). Reproductive investment by both sexes is therefore extensive (Berglund *et al.*, 1986b), allowing for both maternal and paternal effects on offspring fitness. Female reproductive fitness is highly depend-

ent on the care provided by the male (Berglund *et al.*, 1986b), rendering the ability of females to assess paternal quality particularly important. When given the opportunity, females show a clear preference for larger males (Berglund, Rosenqvist & Svensson, 1986a; Berglund *et al.*, 2005). Large males provide better oxygenation during brooding (Braga Goncalves, 2010) and also give birth to larger offspring compared to smaller males (Ahnesjö, 1992a, b). However, it is not clear whether the latter result arises from large males providing better paternal care or whether it is solely the result of larger males gaining preferential access to larger females, which produce larger eggs (Braga Goncalves, Ahnesjö & Kvarnemo, 2011), generating embryos that have faster growth and higher chances of survival (Ahnesjö, 1992b). Yet, despite the well-established preference for larger males, individual females transfer eggs containing 11% more protein when they mate with a small compared to a large male in no-choice experiments (Braga Goncalves *et al.*, 2010). Because proteins in fish eggs are positively correlated with survival to the eyed embryo stage, and hence with egg quality (Lahnsteiner, 2007; Finn & Fyhn, 2010), this result can be interpreted in

light of the reproductive compensation theory (Gowaty *et al.*, 2007; Gowaty, 2008) because females increase their reproductive effort when mating with less-preferred partners (Braga Goncalves *et al.*, 2010).

The present study aimed to assess whether male size affects paternal care quality in terms of embryo size and survival, as measured approximately halfway through the brooding period. Given that small males receive eggs with higher protein content at mating, we expect their offspring to be larger and have higher survival than those of large males, if quality of care does not differ between males (Fig. 1D). By contrast, if large males provide higher quality care than small males, we predict higher offspring growth and survival (Fig. 1B) or no difference (Fig. 1C), depending on whether the initial difference in egg protein content compensates fully or partially for the difference in paternal care.

MATERIAL AND METHODS

ETHICS STATEMENT

The present study was conducted in accordance to Swedish law, with ethical approval given by the relevant authority (i.e. the Swedish Animal Welfare Agency; permit number 196-2005). No other permit was needed for collecting or handling the fish. The study did not involve any endangered or protected species.

HUSBANDRY AND SUBJECTS

The present study was carried out at Kristineberg Marine Research Station, on the Swedish west coast (58°15'N, 11°28'E) between May and July 2006. Pipefish were caught in eelgrass (*Zostera marina*) meadows in the early part of the breeding season with a small beam trawl (4-mm mesh size), pulled behind a boat. Prior to the experiment, all fish were held in large storage barrels (225 L), separated by sex and size for 2–3 weeks. Both barrels and experimental aquaria were provided with artificial eelgrass and a flow-through system of continuously renewed natural seawater. Salinity followed natural conditions under a 16 : 8 h light/dark cycle and a constant temperature of 14 °C. Fish were fed twice daily with cultured newly hatched and older *Artemia salina*, and supplemented with wild caught mysid and common shrimp (*Praunus flexuosus* and *Crangon crangon*, respectively) and copepod spp.

All fish had their standard body length (SL) measurements taken to the nearest mm immediately before starting the experiment. Additionally, female body depth was recorded to the nearest 0.1 mm using callipers across the thickest part of the trunk. Female body depth varied between 7.6 mm and 12.7 mm

(mean \pm SE: 9.3 ± 0.3 mm). Female body length and depth correlated positively (Pearson's correlation $r = 0.60$, $N = 34$, $P < 0.001$), however, neither length nor depth differed significantly between treatments (t -tests: SL: $t = 1.24$, d.f. = 32, $P = 0.22$; body depth: $t = 0.02$, d.f. = 32, $P = 0.98$).

EXPERIMENTAL DESIGN

Syngnathus typhle females have a single germinal ridge in each ovary, producing eggs continuously throughout the breeding season (Sogabe & Ahnesjö, 2011), which they then transfer to the male pouches during mating in relatively small batches. Each female was randomly allowed to mate with one small and one large male (treatment A), or in reversed order (treatment B). Females were chosen to be of relatively large sizes (SL, mean \pm SE: 221.1 ± 3.7 mm, range = 190–267 mm, $N = 34$) to ensure they were sufficiently fecund to fill up the brood pouches of both males within a relatively short time (6–7 days). Breeding pairs remained in the mating tanks until the male pouch was full. Once the first male was fully mated, the male was removed from the tank and the female was left alone until the next day, when the second male was introduced in the mating tank. After having mated to both males, all females were released close to the site of capture. Mated males were transferred into brooding tanks (26 \times 45 \times 40 cm or 26 \times 35 \times 35 cm). The two males that had mated to the same female were kept together, being visually identifiable by their size difference.

After 18 days of brooding, males were euthanized by immersion in 1 mL l⁻¹ 2-phenoxyethanol/seawater solution, followed by severing of the spinal column immediately posterior to the operculum. Each male was preserved in 95% ethanol in individual vials. The brood pouch content of the males was dissected to assess relative embryo mortality by calculating the proportion of nonviable (undeveloped and underdeveloped embryos and burst oocytes) relative to the total number of eggs received. Between three and five viable embryos from each male were removed from different areas of the brood pouch and separated from both the eggshell and the remaining yolk. Pictures of the individual embryos were taken (camera: PowerShot S40, 4 MP; programme: Leica IM 1000, version 1.20, Meyer Instruments) using a light microscope ($\times 100$ magnification; Leica MCFL III, Leica Microsystems Ltd). The total length (± 0.01 mm, tip of rostrum to tip of tail) of each embryo was measured from the photographs and calculated using IMAGE TOOL, version 3.0 (University of Texas Health Science Center). The mean length of these embryos gave us an estimate of embryo size in each brood at experimental termination.

STATISTICAL ANALYSIS

Of the 34 females, five failed to mate with both males. A further nine females did not fill one of the pouches to over half their capacity. These replicates were removed from the analysis. In the remaining replicates ($N = 20$), the small males ranged between 133 and 161 mm body length (SL, mean \pm SE: 147.3 ± 1.6) and the large males between 165 and 208 mm (SL, mean \pm SE: 187.3 ± 2.9). Of the above 40 males, eight large males and 10 small males concluded the 18-day brooding period. The remaining replicates were terminated as a result of male illness or premature death and are only included in the analysis of number of eggs initially transferred into the brood pouches.

Given the difficulty in obtaining paired replicates and the small samples sizes in this study, a linear mixed-model (LMM) approach was applied to test the effects of male size and mating order on number of eggs initially received, relative embryo mortality, and embryo lengths at experimental termination. Female identity was included as a random effect to control for maternal effects in the three pairs of males that had mated to the same females. Hedge's D effect sizes (bias-corrected Cohen's D effect sizes for low sample sizes) with corresponding confidence limits (Nakagawa & Cuthill, 2007) were calculated for the differences in relative embryo mortality and mean embryo length between first and second partners and between large and small males. We used Akaike's information criterion adjusted for small sample sizes (AICc) to select from various covariance structures to estimate the degree of covariance between the treatments. The structure 'variance components' provided the lowest AICc values showing the best fit to our data and was used in all models. SPSS, version 15.0 (SPSS Inc.) was used to perform all statistical analyses. Relative embryo mortality was arcsine square-root transformed to conform to assumptions of linearity (Sokal & Rohlf, 1981). $P \leq 0.05$ was considered statistically significant.

RESULTS

Total number of eggs received was significantly greater for large than small males (LMM: $F_{1,18} = 18.1$, $P < 0.001$; mean \pm SE, small males: 73.3 ± 5.5 eggs, large males: 112.3 ± 7.7 eggs). Females also transferred significantly more eggs into the pouch of their first than second partner (LMM: $F_{1,18} = 5.62$, $P < 0.05$; mean \pm SE, first: 104.9 ± 7.5 eggs, second: 80.7 ± 7.6 eggs) independent of male size class (LMM: $F_{1,18} = 0.34$, $P = 0.57$). Female identity had no effect on the number of eggs transferred to the males (LMM: Wald $Z = 0.38$, $P = 0.70$).

Embryo mortality ranged from 0% to 25% (mean = 8.5%) and was affected both by female iden-

tity (LMM: Wald $Z = 2.05$, $P = 0.04$) and by mating order [LMM: $F_{1,13.77} = 18.40$, $P = 0.001$; mean \pm SE, first: $14.2 \pm 3.1\%$, second: $2.9 \pm 1.4\%$; effect size (ES) = 1.47, lower and upper confidence interval (CI) = 0.33–2.61] (Fig. 2) but, importantly, not by male size class (LMM: $F_{1,13.77} = 0.19$, $P = 0.65$; mean \pm SE, small males: $10.2 \pm 2.9\%$, large males: $5.9 \pm 3.0\%$; ES = 0.44, CI = -0.59 to 1.48). Male size and mating order showed no interaction (LMM: $F_{1,12.20} = 0.45$, $P = 0.51$).

Mean embryo length did not differ between large and small males (LMM: $F_{1,13.45} = 0.55$, $P = 0.47$; mean \pm SE, small males: 3.78 ± 0.22 mm, large males: 4.44 ± 0.43 mm; ES = 0.69, CI = -0.37 to 1.75) and was not affected by female identity (LMM: Wald $Z = 0.052$, $P = 0.96$). There was a tendency for second mates to have larger embryos than first mates (LMM: $F_{1,13.52} = 3.47$, $P = 0.08$; mean \pm SE, first: 3.63 ± 0.28 mm, second: 4.44 ± 0.30 mm; ES = 0.89, CI = -0.16 to 1.95). Male size and mating order showed no interaction (LMM: $F_{1,13.41} = 1.48$, $P = 0.24$).

DISCUSSION

We found an important effect of female mating order, with first mates receiving more eggs and presenting greater levels of embryo mortality. First, higher egg density, resulting from females transferring a greater number of ripe eggs, may negatively affect the ability of a male to osmoregulate and provide nutrients and oxygen for embryo development and survival (Ahnesjö, 1992a). Second, because the females were restrained from mating before the experiment started, our result may also be a result of over-ripened eggs. Over-ripening commonly affects female fertility in fish, with egg quality quickly deteriorating after ovulation (Kamler, 2005). In nature, such time constraints may pressure *S. typhle* females to mate with less-preferred partners. This may be particularly important in the present study population, given the female-biased operational sex ratio that typically arises a few weeks into the breeding season, resulting in limited male availability and intense female-female competition (Berglund *et al.*, 1989; Vincent, Ahnesjö & Berglund, 1994).

We also found a significant effect of female identity on embryo mortality, which shows that there was a maternal effect in the six paired broods. However, because of the small sample size of paired broods, we are unable to draw any further conclusions.

Although it was not measured in the present study, we have good reason to assume that egg protein content differed between eggs passed to small and large males, just as it did in a previous study (Braga Gonçalves *et al.*, 2010). The two studies were carried out in strict parallel: at the same time, in the same

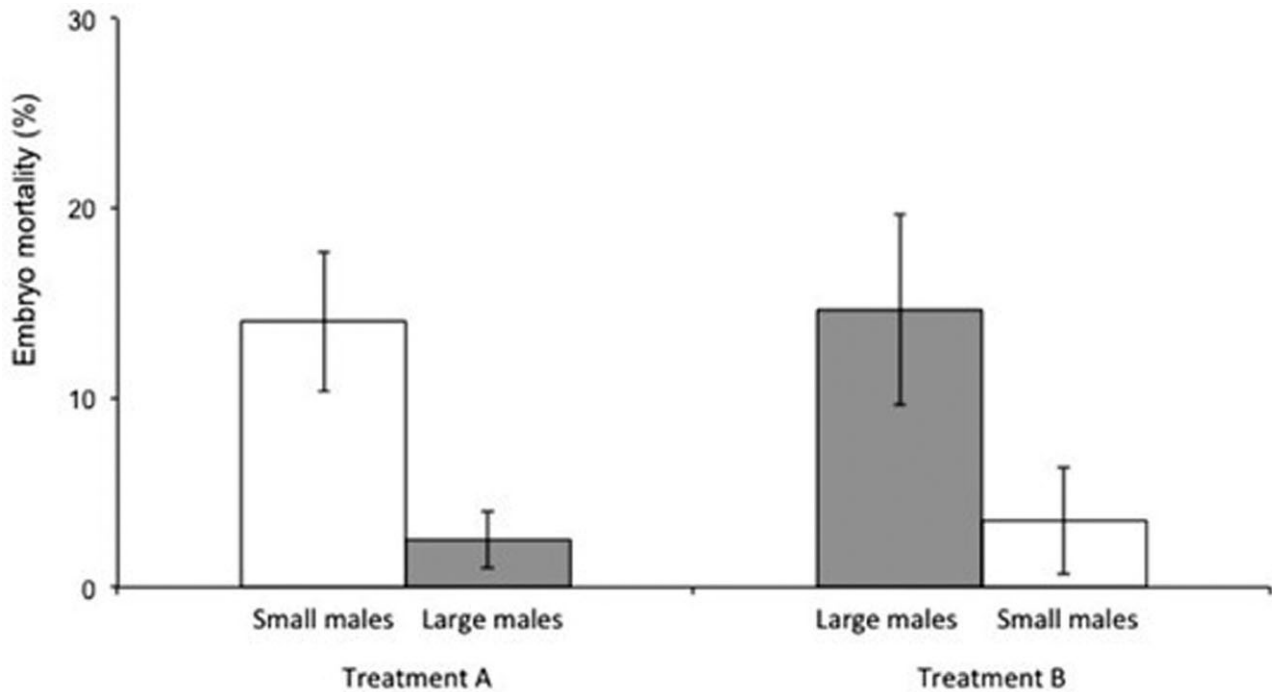


Figure 2. Effects of mating order and male size on embryo mortality in the broad-nosed pipefish. Percentage embryo mortality was measured in broods of small (white) and large (grey) *Syngnathus typhle* males, after a brooding period of 18 days, under two different mating order treatments: (A) female mated to small male first, large male second, (B) reversed mating order. Graph shows mean \pm SE of untransformed data. $N = 18$. Mating order: $P = 0.001$; male size: NS.

aquarium room, using individuals from the same population, kept in the same storage barrels prior to being used in the experiments and given the same feeding regime. The studies differed only in that the zygotes were sampled directly after mating or after 18 days of brooding. Hence, our results showing that relative embryo mortality and mean embryo length did not differ between large and small *S. typhle* males, when measured halfway through the brooding period, suggest that large males provide better nourishment compared to small males. Recent evidence of paternal transfer of amino acids to the developing embryos in this species (Kvarnemo *et al.*, 2011) suggests that there is ample opportunity for variation to arise in male nourishment for offspring development and survival. Similarly, in a closely-related species *Syngnathus scovelli*, male size is strongly and positively correlated with brood survivorship (Paczolt & Jones, 2010). Together with better oxygenation (Braga Goncalves, 2010) and higher survivorship (Sandvik, Rosenqvist & Berglund, 2000), our results help to explain the well-documented female preference for large partners (Berglund *et al.*, 1986a, 2005).

As indicated in Figure 1, the fact that small males receive eggs containing more protein than do large

males (Braga Goncalves *et al.*, 2010) makes the scenario illustrated in Figure 1A unlikely in this species. Furthermore, if we assume that higher initial egg protein content translates into better quality embryos, as has been shown in other fish species (Lahnsteiner, 2007; Finn & Fyhn, 2010), our observation of similar size and survival between embryos brooded by large and small males excludes Figure 1D. Taken together, our results therefore lend support to the reproductive compensation hypothesis (Gowaty, 2008). The fact that we interrupted brooding halfway through the male pregnancy suggests partial (Fig. 1C) rather than full compensation (Fig. 1B) to be a more likely strategy for female *S. typhle*.

In the broad-nosed pipefish, partner size is a strong predictor of egg size in females (Braga Goncalves *et al.*, 2011) and the ability to oxygenate the embryos in males (Braga Goncalves, 2010). Concurrently, it is a major factor for mate choice in both sexes (Berglund *et al.*, 1986a, 2005; Berglund & Rosenqvist, 1990). However, other factors also play a role in mate choice in this species. For example, Sandvik *et al.* (2000) highlighted the importance of olfactory cues, which can provide information with regard to the immune system of potential partners. Moreover, Roth *et al.*

(2012) have shown that both males and females can influence offspring immunity in our study species and also that, in the absence of reliable visual cues, males may use major histocompatibility complex-based cues to select partners (Roth *et al.*, 2014). However, it is not known whether females display similar olfactory-based mate choice. Other factors that may affect mate choice include, for example, body condition (for both sexes) and brood pouch characteristics in males. In the present study, all males were healthy and in good condition at the start of the experiment, although our focus was only body size; thus, in the present study, we are unable to evaluate the potential influence of other factors on female reproductive decisions.

Reproductive compensation and differential allocation have come to be regarded as two ends of a continuum of parental investment tactics (Harris & Uller, 2009; Ratikainen & Kokko, 2010), with differential allocation apparently being more common under a broader range of fitness scenarios. By contrast, reproductive compensation is predicted to occur when baseline offspring survivorship is high, when expected future mate quality is low, and when females cannot forego reproduction (Harris & Uller, 2009). Based on the findings of the present study, we hope to draw attention to the increased likelihood of observing reproductive compensatory investment tactics in species where females compete among themselves for access to mates, where females have little control over partner quality, and may not be able to forego reproduction, and baseline reproductive investment is naturally high as a result of the production of large eggs. Broad-nosed pipefish fall into this category because male mate choice is prevalent; the operational sex ratio becomes more female biased and the quality of potential mates drops throughout breeding season as the best quality males begin brooding their first clutches, and females do not appear to be able to reabsorb their eggs. The results of the present study thus lend support to the reproductive compensation hypothesis.

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