Females increase egg deposition in favor of large males in the rainbowfish, *Melanotaenia australis*

Jonathan P. Evans, Tegan M. Box, Penny Brooshooft, Jack R. Tatler, and John L. Fitzpatrick

Centre for Evolutionary Biology, School of Animal Biology, University of Western Australia, Nedlands, WA 6009, Australia

Females often partition their reproductive investment differentially according to variation in male phenotype. Although evidence for differential maternal investment is accumulating in species with resource-based mating systems, there is relatively little evidence for such effects in species lacking resources at reproduction. In this paper, we evaluate the potential for differential maternal investment in the Australian rainbowfish, *Melanotaenia australis*, an egg scattering freshwater fish with a non-resource-based mating system. We found that female preferences for relatively large males were reinforced through patterns of differential egg deposition that favored preferred males. We also found that females mated first to relatively large males exhibited a reduction in egg deposition when subsequently paired with smaller males, suggesting that patterns of maternal investment can be influenced by recent mating history. We found no evidence that these patterns of differential egg deposition were influenced by male aggression, which did not differ between large and small males. Sexual conflict through physical manipulation is therefore unlikely to account for these patterns. Instead, our findings are more consistent with a pattern of differential allocation, although future studies are needed to evaluate the fitness effects of egg allocation, and the predicted trade-off between current and future reproductive investment. Irrespective of the evolutionary mechanisms underlying these patterns, our results confirm that female rainbowfish exhibit rapid and flexible changes in their reproductive investment according to male sexual attractiveness. Key words: differential allocation, freshwater fish, good genes, mate attractiveness, maternal effects, sexual selection. [Behav Ecol 21:465–469 (2010)]

There is growing evidence that sexual selection can favor flexibility in maternal investment (Sheldon 2000; Harris and Uller 2009). However, predictions about the way females should adjust their reproductive investment differ. For example, the differential allocation hypothesis (DAH) predicts that females should differentially allocate resources at reproduction in favor of relatively attractive males because of the direct or indirect fitness benefits associated with mating with attractive males (Burley 1986). Accordingly, females are expected to bear greater costs and invest more resources in offspring fathered by high-quality males by investing in current reproductive opportunities at the expense of future opportunities (Burley 1986; Sheldon 2000). By contrast, the reproductive compensation hypothesis (RCH) generates the opposite prediction by suggesting that when females have limited reproductive opportunities they should increase their reproductive investment when mating with “low”-quality males (Gowaty et al. 2007). The logic behind this model is that increased investment by females is expected to counteract the negative effects of mate quality on offspring fitness. Recent attempts to model both processes suggest that reproductive compensation will only occur under restricted conditions, unlike differential allocation which is robust across a broad range of biological parameters (Harris and Uller 2009). Finally, hypotheses invoking sexual conflict suggest that plasticity in reproductive investment may be nonadaptive from the female’s perspective, for example, when males harass females into increasing their current investment at a cost to future reproductive opportunities (Arnqvist and Rowe 2005).

Evidence that females invest differentially according to the quality of their mates comes primarily from birds, and some invertebrates, where studies have revealed the effects of male attractiveness on clutch size (e.g., Thornhill 1983; Petrie and Williams 1993; Parker 2003; Galetti et al. 2006), egg size or mass (Cunningham and Russell 2000; Kolm 2001; Gilbert et al. 2006), or egg contents such as the amount of hormones or yolk antibodies sequestered to each egg (e.g., Gil et al. 1999, 2004; Saino et al. 2002). With the exception of studies where females’ reproductive opportunities are constrained (e.g., Gowaty et al. 2007), almost all the available evidence points to increases in maternal investment when mating with attractive (i.e., high quality) males, thus providing support for the DAH. Until now, however, most of the evidence revealing such patterns is confined to species exhibiting parental care, where patterns of female investment are readily explained by direct selection on female mating preferences (e.g., Kokko et al. 2003). By contrast, there are only a few examples of differential reproductive investment by females in species that lack parental care or where males offer no material resources that can directly increase female reproductive fitness. In the resource-free mating system of the zebrafish *Danio rerio*, for example, females lay more eggs in favor of phenotypically attractive (larger) males (Skinner and Watt 2007), whereas in a hybridogenetic waterfrog complex of the genus *Rana*, females release fewer eggs when paired with hybrid males (Reyer et al. 1999). Importantly, Reyer et al. (1999) showed that reductions in clutch size resulted in greater residual reproductive value in the subsequent year, thus providing evidence for a trade-off between current and future reproductive fitness that is explicit to the DAH. In such resource-free
mating systems, differential maternal investment is thought to facilitate the acquisition of indirect (genetic) benefits, for example, if females bias their reproductive investment in favor of males that bestow high genetic fitness on their offspring (e.g., Kotiaho et al. 2003; Parker 2003).

In this paper, we evaluate the potential for differential maternal effects based on variation in male sexual attractiveness in the western rainbowfish, *Melanotaenia australis*, an externally fertilizing freshwater fish endemic to northwestern Australia (Phillips et al. 2009). Although almost nothing is known about the mating behavior of this species in its natural environment, a recent study confirms that females exhibit sexual preferences for relatively large males and that these preferences are reinforced through intrasexual competitive interactions that favor relatively large males in gaining access to mates (Young et al. 2010). In this study, we further examine the potential for female preferences to favor relatively large males but extend this work to evaluate the potential for females to increase egg deposition in favor of relatively attractive males. We also evaluate male and female behavior to determine whether patterns of egg deposition can be determined by aggressive intersexual interactions.

To test these ideas, we employed a paired crossover design to determine whether female rainbowfish differentially regulate their reproductive effort (the number and size of eggs spawned) according to relative male size, thus potentially reinforcing their mating preferences for large males. Because female reproductive investment can potentially be influenced by male sexual harassment, and thus represent a cost of mating with attractive males (i.e., sexual conflict; examples reviewed by Arnbjörn and Rowe 2005), we also took account of male–female aggressive interactions during the mating trials in an attempt to evaluate whether such interactions are associated with female egg laying behavior. Our experimental design enabled females to observe 2 males differing in body size before allowing them to mate with one of the males (either large or small). We then repeated the procedure so that each female again viewed the same 2 males before being paired with the second male. In this way, each female had the opportunity to mate with both males sequentially, and the order of presentation of small (large or small) was reciprocated so that half of females interacted first with the large male and half interacted first with the small male.

**MATERIALS AND METHODS**

**The study species and its maintenance**

The fish used in this experiment were captured in 2006 from a natural population inhabiting a tributary of the Fortescue River near Wittenoom, Western Australia (lat 22°14.89′S, long 118°19.44′E). Fish were returned to aquarium facilities at University of Western Australia where they were maintained in mixed-sex aquaria (90 × 50 × 50 cm, filled to 27 cm) at 24°C on a 9:15 h light:dark cycle. All fish were fed a mixed diet of from bloodworm and live *Daphnia* nauplii. Like other melanotaenids, *M. australis* exhibit a “trickle spawning” manner that under stable conditions they will breed year-round, with females scattering a small number of eggs daily (Allen and Cross 1982; Allen 1995). The eggs are deposited among vegetation (or artificial mops, see below) and are abandoned shortly after spawning by both parents. In the captive population used for this study, we have consistently isolated fertile eggs from spawning mops on a daily basis and are therefore confident that the females selected for use in our experiment were sexually receptive at the time of testing. Furthermore, we ensured that we only used females that were larger than 50 mm in body length (distance from the snout to the tip of the caudal peduncle [=standard length, SL]; mean ± standard error [SE] = 59.4 ± 0.6 mm; range 52–65 mm). This is the size at which female melanotaenids consistently shed eggs and maintain daily spawning activity (Ivantsoff et al. 1998).

**Experimental design**

We conducted 36 paired replicates, each involving 2 males and a single female. Adult females were taken at random from experimental stocks on the provision that they were sexually mature (see above). Males, on the other hand, were selected for each replicate on the basis of differences in body size. Within each of the n = 36 pairs of males, the minimum difference in SL between large and small males was 4 mm (range 4–20 mm), and these differences were highly significant (large males: 63.6 ± 0.5 mm; small males: 53.9 ± 0.5 mm; independent samples *t*-test: *t* 0.05 = 13.8, *P* < 0.0001).

The entire experiment was conducted in 3 blocks of 12 replicates. Within each block, we used 12 replicate tanks (44 × 42 × 34 cm, filled to 20 cm), each containing 2 circular plastic containers (21 capacity, 12 cm diameter, and perforated with 12 holes of 5 mm diameter) positioned on opposite sides of the tank. On the evening before the first trial the small and large male within each pair were placed individually into each plastic container and a female was placed into the center of the tank. The fish were allowed to settle overnight before we observed the female’s behavior the next morning (from 9:00 h). During these observations, we recorded the time that each female spent within one body length of each male’s container over a 10-min period. Females were then left in the tank for 4 days with visual and olfactory, but not direct, access to the 2 males. On day 4 of the trial (16:00 h), either the large or small male was released from his plastic enclosure into the main tank. The order of presentation of males (large or small) was reciprocated so that half of females interacted first with the large male and half interacted first with the small male. The containers of released males were removed from the tank and replaced with a spawning mop constructed of 20 strands of 20 cm dark green wool attached to a cork buoy. These spawning mops were weighted with a steel bolt so that they floated 8 cm from the bottom of the tank. The released male was then allowed to interact freely with the female over the next 4 days. During this period (days 5–8), we removed the spawning mop every afternoon (14:00 h) to collect spawned eggs, which were retrieved by combing each strand of the mop with forceps. Collected eggs were counted and photographed along with a section of a stage micrometer (Wild 310345) under ×40 magnification (Olympus BX41). Egg diameter was estimated from these photographs using ImageJ (National Institutes of Health, Bethesda, MD). In some cases (n = 6 females), egg diameter measures were not taken because eggs had been damaged during the retrieval process. This cycle was repeated throughout days 9–16 (period 2) with the alternate males to provide paired data for female behavior, egg counts, and size measures.

During periods of direct male–female interactions (days 5–8 and 9–16), we performed daily 10-min focal behavioral observations between 9 am and 12 pm and recorded male courtship and aggressive behaviors toward females. To assess courtship behaviors, we recorded and summed the frequency of head shakes (side-to-side head movements), fin displays (full extension of the dorsal, pectoral, and pelvic fins), and rushes (rapid movement toward the female). We considered chases (pursuing the female around the tank) and bites (biting the female’s body) as aggressive behaviors. For each male, behavioral observations were analyzed using average daily values.

**Statistical analyses**

To analyze female preferences (i.e., association times), we converted each female’s time scores (time spent with L and S
males) into a single standardized measure, using the formula: \[(L_t - S_t)/L_t + S_t\]; where \(L_t\) and \(S_t\) represent the time (in seconds) that females spent with the large and small males, respectively (Stoner and Breden 1988). Positive values of these scores indicate association times in favor of large males. We then used a one-sample \(t\)-test on these values to test whether their mean value significantly deviated from zero. Paired tests (Wilcoxon signed-rank or \(t\)-tests) were used to assess if male courtship and aggressive behaviors differed between pairs of large and small males. Next, we used linear mixed-effects models to analyze treatment effects (male size category), period (first and second matings), and their interaction on the traits of interest (Díaz-Uriarte 2001, 2002). In these models, we included female ID (subjects) and block (3 levels) as random effects (Díaz-Uriarte 2001) and female body size (SL) as a covariate. We also tested for a mating sequence-by-period interaction to determine whether mating order during the first mating period (either L-S or S-L) influenced subsequent patterns of reproductive investment. To avoid overparameterization of the original \(2 \times 2\) crossover model (Díaz-Uriarte 2002), we performed separate linear mixed-effects models to test for sequence-by-period interactions. In these analyses, we included mating sequence and mating period, along with their interaction, as fixed effects, and female ID and block as random effects. The sample sizes for the mixed-models (\(n = 32\) paired trials) were lower than those for the female choice trials (\(n = 36\) because we had to exclude 4 replicates due to excessive algal grown in 4 tanks during the latter phases of the experiment (i.e., period 2). An algal cure treatment (\(3 \text{ g} \cdot \text{L}^{-1} \cdot \text{2-chloro-4, 6-bis-[ethylamino]s-triazine; Aquamaster}\) was used to control the algae, but this may have influenced female spawning behavior (no eggs were found in these tanks after the application of the treatment). However, the inclusion of data from these replicates in the linear mixed-effects models did not change our findings (indeed they strengthened the egg number effect—see RESULTS).

**RESULTS**

Our analysis revealed highly significant preferences by females in favor of large males during the initial behavioral observations. On average, females spent almost 70% more time associating with large males than with small males (mean ± SE association times: time [in seconds] associating with large males: 99.3 ± 12.6; small males: 59.4 ± 8.0; one-sample \(t\)-test: \(t_{59} = 3.99, P < 0.0001\)). We also found significant treatment effects on the number of eggs deposited (analyses summarized in Table 1; see also Figure 1). When egg count data were pooled across both mating periods, females produced almost twice as many eggs when paired with the larger males (mean number of eggs ± SE = 14.38 ± 1.23) compared with when they were paired with smaller males (7.81 ± 2.77). We found no evidence that this effect was due to a difference in the number of days that females laid eggs between the treatment (mean ± SE number of days that females laid eggs: small male treatment: 2.22 ± 0.22; large male treatment: 2.31 ± 0.25; treatment effect in linear mixed-effect model: \(F_{1,30} = 0.20, P = 0.89\)). This confirms that females either adjust the number of eggs per spawning event or the total number of spawnings performed with a given male over the 4-day period.

We found that females partitioned their eggs irrespective of male courtship and aggressive behaviors, which did not differ between large and small males (mean courtship ± SE [range]; large males: 34.9 ± 4.3 [8.75–107], small males: 39.8 ± 5.3 [4–127], \(t_{52} = -0.70, P = 0.49\); mean aggression ± SE [range]; large males: 4.4 ± 0.8 [0.25–18.5], small males: 3.1 ± 0.5 [0–9.5], Wilcoxon signed-rank test, \(S = 56.5, P = 0.25\)). Thus, neither courtship nor aggressive interactions between males and females appeared to influence female egg deposition behavior. Mating period also had a significant effect on egg number, with the number of eggs deposited dropping significantly across both treatments in the second mating period (Table 1; Figure 1). Nevertheless, the effects of treatment were consistent across both mating periods, as evident from the nonsignificant treatment-by-period interaction (Table 1; Figure 1). By contrast, we found no significant effects of either treatment or period on egg size (Table 1) and no significant association between mean egg size and egg number during either period (Pearson’s correlations: period 1: \(r = 0.08, n = 31, P = 0.66\); period 2: \(r = 0.16, n = 27, P = 0.44\)).

We also found that patterns of egg deposition during period 1 influenced subsequent patterns of investment in the second

<table>
<thead>
<tr>
<th>Fixed effects</th>
<th>Egg number</th>
<th>Egg size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>(P = 0.04)</td>
<td>(P = 0.90)</td>
</tr>
<tr>
<td>Period</td>
<td>(P = 0.30)</td>
<td>(P = 0.09)</td>
</tr>
<tr>
<td>Treatment × period</td>
<td>(P = 0.28)</td>
<td>(P = 0.56)</td>
</tr>
<tr>
<td>Female SL</td>
<td>(P = 0.04)</td>
<td>(P = 0.15)</td>
</tr>
</tbody>
</table>

**Table 1**

Results from linear mixed-effects models analyzing the effect of treatment (large or small males) and mating period (repeated measures) on egg number and egg size in *Melanotaenia australis*.

![Figure 1](https://example.com/figure1.png)

Figure 1

Number of eggs produced by female *Melanotaenia australis* over a 4 day period when paired with large (open circles) and small (filled circles) males. The crossover design involved pairing each female with large and small males on 2 consecutive occasions (periods 1 and 2, in boxes), with females paired with large males first or small males first in half of the trials. In the left-hand panel circles represent individual responses for each female, whereas in the right-hand panel the circles depict mean (±SE) responses.
mating period and that these patterns depended on mating sequence in the first mating period (linear mixed-effects model: sequence-by-period interaction \( F_{1,30} = 5.48, P = 0.026 \)). Overall, clutch sizes did not significantly differ between females allocated to the LS and SL groups (mean number of eggs \( 24.35 \pm 5.24 \); SL: 19.75 \( \pm 3.70 \); independent samples \( t \)-test: \( t_{60} = 0.12, P = 0.488 \)), but the way that females allocated this fixed pool of eggs depended on the order in which they encountered the 2 males.

**DISCUSSION**

Our study reveals that female *M. australis* produce larger egg clutches for larger males, supporting a small but growing number of studies reporting similar patterns of female investment in relation to male attractiveness in fishes (Hastings 1988; Katano and Maekawa 1997; Kolm 2001; Skinner and Watt 2007). However, our study is one of just a handful that have experimentally tested for such effects in species lacking parental care or other resources that directly impact female fitness (see also Reyer et al. 1999; Skinner and Watt 2007).

Our results offer no support for the RCH, which predicts that females should increase their reproductive investment when mating with low-quality males (Gowaty et al. 2007). Instead, they are more easily interpreted in the context of adaptive differential allocation (Burley 1986). However, an equally plausible scenario to account for these results is that females are manipulated into increasing their reproductive investment toward larger males, resulting in a direct cost for females (Arnquist and Rowe 2005). Recent evidence for such effects comes from the bruchid beetle *Calllosobruchus maculatus*, where male sexual harassment results in females laying fewer eggs and ultimately producing fewer offspring (Gay et al. 2009). Although we found no evidence that females assigned to the different treatments differed in the level of harassment they endured, egg deposition dynamics can be influenced by more subtle effects. For example, Arnquist and Rowe (2005) argued (with some supporting evidence) that male display traits may evolve to exploit and manipulate female reproductive investment above the naturally selected optimal levels (p. 103–106). Clearly we cannot rule out such conflicts of interest in this study, and a crucial next step toward understanding the potential evolutionary significance of these findings is to evaluate the fitness consequences of mating with large males, along with the potential long-term fitness costs of increased egg allocation.

We also found that, overall, clutch sizes were similar between females allocated to both mating sequences. This suggests that females had a fixed number of eggs available when they entered the trials, independent of the treatment group to which they were first assigned. However, the way that females allocated their eggs was influenced by prior mating history. Females that encountered large males first deposited a high proportion of their egg resources to their initial matings, resulting in a reduction in clutch size during the second mating period (when they encountered the small male). This contrasted with the observed patterns of egg allocation by females assigned to the SL sequence, where the increase in clutch sizes during the second period was only marginal, suggesting that the females’ ability to increase their reproductive investment toward high-quality males was constrained by the number of eggs that they had previously allocated toward their first (small) mating partners. Thus, over short periods at least, females appear to be constrained in the number of eggs laid by their previous investment. Much stronger evidence for a trade-off between current and subsequent reproductive investment was reported by Reyer et al. (1999), who showed that in waterfrogs females adjusted their clutch size by releasing fewer eggs when amplexed with genetically incompatible males. Reyer et al. (1999) addressed the long-term consequences of such differential investment by measuring the same females’ reproductive output over successive years. Females that released fewer eggs in the first reproductive bout were able to release more eggs the following year.

We found no evidence for a relationship between egg size and egg number or an effect of treatment on the size of eggs produced (cf. Cunningham and Russell 2000). This may be because the time frame of our study was insufficient for females to alter the size of their eggs. Alternatively, egg size may be relatively inflexible in this species. In future, we intend to evaluate the potential for such effects and look for trade-offs between egg number and size by conducting mating trials over relatively longer periods. This would also enable us to assess possible long-term fitness consequences of increased reproductive “investment,” for example, through long-term reductions in fecundity in females with initially high levels of reproductive investment (e.g., Reyer et al. 1999).

In summary, we have shown that female preferences for large males are potentially reinforced through increased investment in clutch sizes that favor preferred males. These findings therefore support the results from a recent study on *D. rerio* (Skinner and Watt 2007), another species of freshwater fish with a resource-free mating system, by revealing that females are able to differentially partition their available egg reserves according to male size. However, our data go further by indicating a potential trade-off in reproductive investment over a very protracted period (cf. Reyer et al. 1999). Our results also suggest that these effects were independent of aggressive interactions between males and females, which did not differ between the 2 male size classes. In order to complete our investigations of phenotypic plasticity in clutch size in this species, we need to identify the selective basis for female preferences for large males, which may include the acquisition of indirect (genetic) benefits that increase offspring viability (e.g., Reynolds and Gross 1992).

One obvious direction for future research would therefore be to evaluate the potential for indirect fitness gains to arise from mating with large males and to determine whether such benefits are amplified through patterns of differential investment uncovered by our study (e.g., Kotiaho et al. 2003). Fortunately, externally fertilizing species such as *M. australis* offer excellent models for partitioning sources of variance in offspring fitness among genetic (e.g., intrinsic sire effects due to paternal “good genes”) and nongenetic effects, for example, arising from differential maternal effects (Welch et al. 1998; Barber et al. 2001; Wedekind et al. 2001; Pitcher and Neff 2006; Evans et al. 2007). We therefore foresee excellent opportunities for identifying the evolutionary basis for these results in future work.

**FUNDING**

The Australian Research Council (DP0772498 to J.P.E.); Natural Sciences and Engineering Research Council of Canada (to J.L.F.); University of Western Australia (to J.L.F.).

We thank Cameron (Meatball) Duggin for assistance with maintenance and husbandry, Ramón Díaz-Uriarte for statistical advice, and 2 anonymous reviewers for helpful suggestions that improved manuscript. This work was carried out under University of Western Australia’s Animal Ethics approval 05/100/513.

**REFERENCES**

Evans et al. • Females increase egg deposition in favor of large males

Gil D, Leboucher G, Lacroix A, Cue R, Kreutzer M. 2004. Female canaries produce eggs with greater amounts of testosterone when exposed to preferred male song. Horm Behav. 34:64–70.
Pitcher TE, Neff BD. 2006. MHC class IIB alleles contribute to both additive and non-additive genetic effects on survival in Chinook salmon. Mol Ecol. 15:2357–2365.