

## Original Article

Male choice of mates and mating resources  
in the rose bitterling (*Rhodeus ocellatus*)Mara Casalini,<sup>a</sup> Martin Reichard,<sup>b</sup> André Phillips,<sup>a</sup> and Carl Smith<sup>a,b</sup><sup>a</sup>School of Biology, University of St Andrews, Gatty Marine Laboratory, East Sands, St Andrews, Fife KY16 8LB, UK and <sup>b</sup>Institute of Vertebrate Biology, Academy of Sciences of the Czech Republic, Květná 8, 603 65 Brno, Czech Republic

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The traditional view of sexual selection acting wholly through male–male competition and female choice has been challenged in recent years. An increasing body of experimental work has demonstrated a role for male choosiness over mates, influenced by cues, such as female body size, that correlate with fecundity. In addition, in resource-based mating systems, male preference for resources required for reproduction is predicted to match those of females. Using the rose bitterling, *Rhodeus ocellatus*, a fish that uses living freshwater mussels for oviposition, we investigated male response to females that varied in size and to oviposition sites that varied in quality. Male courtship behavior directed at females, and aggression directed at rivals, did not vary with female size. The lack of predicted male response, which contrasts with other species, was explained by the absence of a relationship between female size and either batch fecundity or egg size, stemming from constraints on these variables imposed by oviposition in the gill cavities of mussels. When presented with spawning sites that contrasted in quality, male preferences for spawning sites were inconsistent: males lacked a preference for high-quality sites when no female was present but showed strong preferences for high-quality sites that matched the preferences of females. These results indicate constraints on the evolution of male mate choice and divergence in selection acting on the sexes in making oviposition decisions.

**Key words:** body size, fecundity, male mate choice, mating system, oviposition, sperm competition, territoriality.

## INTRODUCTION

Sexual selection operates primarily through male–male competition and female mate choice (Darwin 1871; Andersson 1994). The conventional view is that to maximize their reproductive fitness, males should mate with the greatest possible number of females, either by monopolizing females themselves or the resources that females require for reproduction. Given their lower potential reproductive rate, the prediction for females is that they should be choosy over mating partners with respect to the resources they provide, their fertility, or their genetic quality (Neff and Pitcher 2005). In consequence, mate choice research has tended to focus on females, attempting to understand the significance of direct and indirect benefits of mate choice (Andersson 1982; Thornhill 1983; Eberhard 1996), and the cues and signals on which females base their choices (Andersson 1994; Searcy and Nowicki 2005).

However, an increasing body of experimental work has recognized a role for male mating preferences for females (Clutton-Brock 2009; Rosvall 2011; Stockley and Bro-Jørgensen 2011). In addition to choosing among females on the basis of fecundity or offspring viability, males may also differentiate among resources that females require for reproduction. Thus, males that guard the best feeding,

oviposition, or nesting sites or the ones that possess the largest territories tend to have the highest reproductive success (Davies and Lundberg 1984; Searcy and Yasukawa 1995; Pärt 2001). The role of male monopolization of resources needed by females for reproduction is well understood (Andersson 1994) and is a key aspect of mating system evolution (Shuster and Wade 2003). Because these breeding resources influence male fitness and may vary in quality, female preferences for breeding resources should be matched by male discrimination of the same resources, with the prediction that males should exercise selectivity over their defense of breeding resources that correspond with the resource preferences of females. Choice of nest or oviposition sites by males, particularly in species with paternal care, is well understood (Clutton-Brock 1991; Andersson 1994).

We tested the role of male mate choice and resource choice using the rose bitterling, *Rhodeus ocellatus*, a small freshwater fish that lays its eggs in the gills of living unionid mussels. Male *R. ocellatus* compete for territories around mussels and actively court females, which they “lead” to mussels (Wiepkema 1961; Kanoh 1996; Smith 2011). Males that control access to mussels enjoy high reproductive success (Reichard et al. 2004, 2005). Male dominance is determined by body size (Reichard et al. 2008; Casalini et al. 2009), with smaller males displaying alternative nonguarder tactics associated with sperm competition (Spence et al. 2013). These roles are not fixed and male mating behavior is flexible and

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highly opportunistic (Kanoh 1996, 2000; Smith et al. 2007, 2009). Females develop long ovipositors that they use to place a clutch of 1–8 eggs in the gill chamber of a mussel in a single spawning act, with females capable of spawning several clutches each day (Pateman-Jones et al. 2011). Males release sperm over the inhalant siphon of the mussel and fertilized eggs develop inside the mussel gill where they are protected and ventilated until development is complete.

Female rose bitterlings are conspicuously choosy about which mussels they will use for oviposition, preferring mussels that do not already host eggs and embryos, thereby ensuring higher egg and embryo survival (Smith et al. 2000; Reichard et al. 2006; Reichard, Le Comber, et al. 2007). Female oviposition decisions represent a key mating decision with direct fitness consequences for the female (Agbali et al. 2010, 2012) and implications for population dynamics (Przybylski et al. 2007; Casalini et al. 2010; Konečná et al. 2010). Dissolved oxygen concentration in the exhalant current is the probable cue used by females to assess mussel quality (Smith et al. 2001), and suffocation or ejection from the mussel is the primary cause of embryo mortality (Kitamura 2005; Spence and Smith 2013). Although some bitterling species are host specialists, rose bitterlings are host generalists, though they have distinct preferences for specific mussel species, with preferences relating to mussel ventilation rates (Reichard, Liu, et al. 2007). Although females display marked mussel preferences, the mussel preferences of males have never been formally tested, though they also intensively inspect the siphons of mussels prior to spawning.

Female bitterlings are also choosy about mates. Mate choice decisions are based on olfactory cues that identify male genetic compatibility (sensu Neff and Pitcher 2005), such that females spawn preferentially with males with major histocompatibility complex alleles complementary to their own. Female mate preferences are adaptive, with significant differences in embryo survival rates between embryos fathered by genetically compatible and incompatible males (Agbali et al. 2010; Reichard et al. 2012). Female mate preferences are influenced by male courtship rate and nuptial coloration (Casalini et al. 2009). Males respond to females with extended ovipositors with courtship behavior, though the extent to which males exercise mate choice, if at all, is unclear. Under natural conditions, territorial male bitterling mates with a succession of females over the course of a day; thus, mate choice is sequential rather than simultaneous (Smith et al. 2009). However, males risk sperm depletion, with the size of ejaculates declining both over successive ejaculations and over successive spawnings (Smith et al. 2009; Pateman-Jones et al. 2011). This cost to sperm production is predicted to generate selection on males to mate preferentially with females that spawn more or higher quality eggs.

To examine the role of male preference for females and oviposition sites, we conducted 3 experimental studies. We tested whether males invested differentially in female size classes, measured in terms of courtship directed toward females and defense directed at a rival (Study 1). In addition, we tested whether female size correlated with clutch size and egg volume (Study 2). Finally, male oviposition site preferences were examined by presenting males with mussels that varied in quality as sites of incubation, in the presence of a female that was either able or unable to make her oviposition site choice (Study 3). We predicted that 1) males would invest more energy in courtship and defense of larger females; 2) female size would correlate positively with egg volume and clutch size; and 3) male preferences for oviposition sites would correspond with those of females.

## MATERIALS AND METHODS

### Study subjects

Fish used for experimental work were first-generation *R. ocellatus* derived from wild-caught fish from the River Yangtze Basin, China. During the experiment, they were 24–36 months old. Prior to experiments, fish were held in stock aquaria measuring 120 (length) × 40 (width) × 45 (depth) cm. Stock and experimental aquaria were on a recirculating system at 23 °C, exposed to 16:8 h light:dark cycle. The recirculating system has high volume (2500 L), with water filtered through a series of mechanical filter bags, fluidized sand filter towers, regassing/trickling bio-tower, and 330 W commercial ultraviolet sterilizer. Any biologically active olfactory cues that might affect fish behavior were unlikely to escape this degree of filtration and would, in any case, be substantially diluted. Fish were fed a mixture of commercial dried fish flake food and bloodworm (*Chironomus* spp.) twice daily and live zooplankton (*Daphnia* spp.) 3 times each week. Freshwater mussels used in trials were *Unio pictorum*, which is the preferred species of mussel used by *R. ocellatus* (Casalini et al. 2009). Mussels were collected from the River Cam, kept outdoor in 100-L tanks and fed with live phytoplankton daily.

### Study 1: male mate preferences

To test male responses to differently sized females, a total of 42 males and 42 females were randomly selected from stock aquaria. Females were assigned by eye to 2 size categories, large and small. Females were divided into 2 size classes to simplify the subsequent analysis. There was a significant difference in body length between the 2 female size classes (unpaired *t*-test, square-root-transformed data:  $t_{40} = 18.06$ ,  $P < 0.001$ ), which varied in age. The mean body length of large females was 53.0 ( $\pm 0.64$  standard error [SE]) mm and small females was 39.7 ( $\pm 0.42$ ) mm. A pair of size-matched males were randomly selected and placed in an experimental aquarium measuring 60 (length) × 40 (width) × 40 (depth) cm containing a mussel in a sand-filled transparent plastic cup and artificial plants as refuges. Opaque barriers were placed between adjacent aquaria to prevent visual interaction between fish. One male always established dominance, usually within 1 h, with dominance unmistakable from the male's elevated display rate and monopolization of the mussel. The display rate of the dominant male to its rival was scored for 15 min using a palm computer with the FIT-system behavior recording software (Held and Manser 2005). Displays comprised finspreading, parallel swimming, and headbutting (see Smith et al. 2004 for full descriptions). After covering the mussel with a transparent perforated plastic cup to allow mussel inspection but not spawning, either a large or small female with an extended ovipositor was randomly selected and gently released into the experimental aquarium and allowed to settle for 1 h. The mussel was then uncovered and the behaviors of the dominant male were scored for a further 15 min. The behaviors scored were 1) displays directed at rival, 2) duration of courtship behavior (Smith et al. 2004), and 3) ejaculation rate. After completion of the trial, female body length was measured to the nearest 1 mm and the female was not used again in the study.

The same procedure was repeated with the same pairs of males and mussel but with a female from the alternate size class to the first tested. The order of testing differently sized females was randomized prior to the beginning of the experiment. After completion of the trial, the body length of both males and of the second female were measured to the nearest 1 mm and were returned to

stock aquaria and not used again. A total of 21 independent paired replicates were completed.

### Study 2: female size and reproductive traits

To test the correlation between female size and egg and clutch size, 32 males were haphazardly selected from stock aquaria and housed in an aquarium measuring 60 (length) × 40 (width) × 40 (depth) together with 16 females with extended ovipositors ranging in body length from 37 to 57 mm. Haphazardly selected pairs of males were placed in a smaller aquarium measuring 25 (length) × 40 (width) × 30 (depth) cm with a mussel in a sand-filled plastic cup and a randomly selected female and left to spawn. Two males were used to maximize the likelihood that females would spawn (Smith and Reichard 2005). After 1 h, the mussel was inspected by gently opening a 1-cm gap between the valves using a mussel-opening device (Kitamura 2005). If eggs were present, the mussel was measured and dissected and the eggs counted and photographed against a scale bar using a Canon EOS 350D with a 60-mm macro lens under standardized light conditions. Because females spawn their eggs in small clutches of 1–8 eggs, and these lie together in the interlamellar spaces of the gill demibranchs, clutch size from separate spawning can be readily established, unless the gills are packed with eggs (>100) so that clutches accumulate on top of each other, which was not the case here. If eggs were not present, the mussel was replaced in the aquarium and examined at hourly intervals until spawning had occurred. Once spawning had occurred, female body length was measured to the nearest 1 mm and the fish and the mussel were not used again in trials.

The diameter of the major and minor axes of all eggs was measured to the nearest 0.1 mm from digital images using the UTHSCSA Image Tool 3.0. Egg volume was calculated using the function for ellipsoids of

$$V = \frac{4}{3}\pi\left(\frac{1}{2}a\right)\left(\frac{1}{2}b\right)^2 \quad (1)$$

where  $V$  is egg volume and  $a$  and  $b$  are the lengths of the major and minor axes, respectively (Coleman 1991). A total of 16 independent replicates were completed.

### Study 3: male spawning site preferences

To examine male oviposition site preferences, a single male rose bitterling was randomly selected from stock aquaria and placed in an aquarium measuring 60 (length) × 30 (width) × 30 (depth) cm with artificial plants as refuges. Two mussels, 1 *Anodonta anatina* and 1 *U. pictorum*, each in a sand-filled plastic cup, were placed at each end of the aquarium, with the position of each species randomized among trials. Given a choice between these 2 mussel species, female *R. ocellatus* show a strong preference for *U. pictorum* (Casalini 2007). Once the male had established territoriality around the mussel, a female confined in a perforated transparent bottle was placed equidistant between the mussels. A male will perform all the normal courtship and leading behaviors to a female confined in this way, even though she is unable to follow him. Once the male started to court her, his behaviors were recorded for 10 min. The behaviors recorded were 1) the frequency that the male inspected the siphons of each mussel and 2) the frequency the male attempted to lead the female to each mussel. Leading behavior involves the male circling in front of the female while beating his caudal and median fins and undulating his body at high frequency and low amplitude, then swimming slowly toward a mussel. If the female fails to follow the

male, he ceases the behavior and swims back to her and resumes the behavior (Smith et al. 2004). After 10 min, the female was gently freed and male behavior and the frequency of female mussel inspection recorded for a further 10 min. At the end of each trial, male and female body lengths were measured to the nearest 1 mm and fish and mussels were not used again. A total of 16 independent replicates were completed.

### Data analysis

All data were tested for normality using a Shapiro–Wilk test and for equality of variance using Bartlett's test. Data that did not meet assumptions of normality and homoscedasticity were transformed to meet these assumptions. In Study 1, an unpaired  $t$ -test was used to test the difference in body length between the 2 female size classes. A 2-way ANCOVA was used to test for differences in the aggression rate of dominant males before and after they were presented with a female and when presented with either large- or small-sized females. Male body size was used as a covariate. A paired  $t$ -test was used to test for a difference in leading rate and ejaculation rate when males were presented with either large- or small-sized females. In Study 2, Pearson's correlation was used to test the correlation between female size and clutch size and mean egg volume. In Study 3, a paired  $t$ -test was used to compare male inspection and leading rates with the 2 mussel species when females were constrained and unconstrained and to test for a difference in female mussel inspection of the 2 mussel species. Mussel shell length was measured in all 3 studies but had no impact on any experimental outcomes and these data are not presented.

## RESULTS

### Study 1: male mate preferences

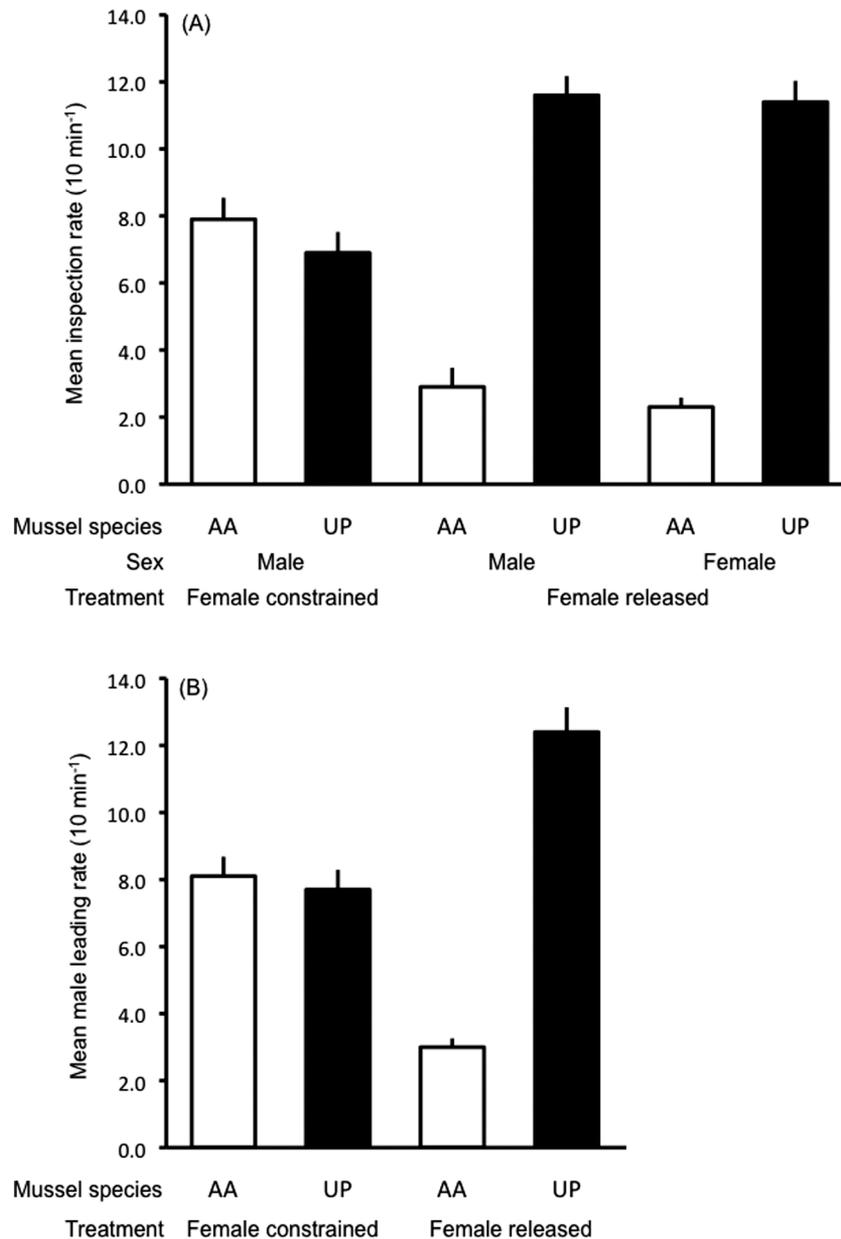
When males were exposed to different-sized females, there was no difference in male display rate (paired  $t$ -test:  $t_{20} = 1.54$ ,  $P = 0.138$ ), in the rate of courtship (paired  $t$ -test, square-root-transformed data:  $t_{20} = 0.43$ ,  $P = 0.670$ ), or ejaculation (paired  $t$ -test,  $\log_{10+1}$ -transformed data:  $t_{20} = 0.56$ ,  $P = 0.585$ ). There was a significant difference in male display rate before and after presentation with a female, irrespective of female size (paired  $t$ -test, square-root-transformed data, small females:  $t_{20} = 5.18$ ,  $P < 0.001$ ; large females:  $t_{20} = 4.57$ ,  $P < 0.001$ ); in each case, the aggression rate increased after introduction of the female. Male size did not correlate with any male behavior (Pearson's correlation: all  $P > 0.05$ ).

### Study 2: female size and reproductive traits

There was no significant correlation between clutch size and either female size (Pearson's correlation:  $r_{14} = 0.13$ ,  $P = 0.637$ ) or mean egg volume ( $r_{14} = 0.20$ ,  $P = 0.451$ ). Mean clutch size was 2.9 ( $\pm 0.28$  SE) eggs, which is typical for *R. ocellatus* (Pateman-Jones et al. 2011), and mean egg volume was 2.3 ( $\pm 0.11$  SE) mm<sup>3</sup>.

### Study 3: male spawning site preferences

When females were constrained, the rate of male inspection of *A. anatina* was not significantly higher than that of *U. pictorum* (paired  $t$ -test,  $t_{15} = 1.92$ ,  $P = 0.074$ ; Figure 1A), though there was no difference in the rate they attempted to lead constrained females to either mussel species (paired  $t$ -test,  $t_{15} = 0.64$ ,  $P = 0.528$ ; Figure 1B). After release of the female, the rate of mussel inspection and the rate of male leading behavior directed at *U. pictorum*



**Figure 1**

Response of male *Rhodnius ocellatus* to 2 species of mussel oviposition sites: *Anodonta anatina* (AA; white bars) and *Unio pictorum* (UP; black bars). Mean rate of male and female inspection of mussels when the female was either constrained or allowed to freely inspect mussels (A), and rate of male leading females to mussels when the female was either constrained or released (B) during a 15-min observation period. Error bars are 1 standard error of the mean.

was significantly greater than toward *A. anatina* (paired *t*-test, mussel inspection:  $t_{15} = 16.15$ ,  $P < 0.001$ ; leading:  $t_{15} = 14.20$ ,  $P < 0.001$ ; Figure 1A,B). All females inspected both mussels at least once, though female inspection rate was significantly greater for *U. pictorum* than for *A. anatina* (paired *t*-test, square-root-transformed data,  $t_{15} = 22.69$ ,  $P < 0.001$ ).

## DISCUSSION

It has been increasingly recognized that male mating preferences for females may play a role in a range of mating systems (Parker 1983; Andersson 1994; Clutton-Brock 2009; Stockley and Bro-Jørgensen 2011). In many invertebrates, fishes and amphibians, female body size correlates with clutch size and egg quality, and

a number of studies have demonstrated greater male investment in larger females (Andersson 1994; Wootton 1998; Bonduriansky 2001). Mating can also incur costs for males, thereby limiting the number of females with which they can mate (Andersson 1994; Bonduriansky 2001). However, the lack of a male preference for large females is not unusual, even when fecundity and size correlate, and has been demonstrated. In the agile frog (*Rana dalmatina*), the risk of lost matings and heterospecific matings in a simultaneous mate choice design was assumed to account for the failure of males to select among females on the basis of size (Hettyey et al. 2005). In the case of *R. ocellatus*, total female fecundity is correlated with body size (Kitamura 2005, 2006). However, this study demonstrated that individual clutch size is not correlated with body size. Further, because females distribute their eggs among a number of

males and oviposition sites, greater investment by males in attracting larger females will not result in greater male reproductive success. Thus, low variation in female batch fecundity may limit the evolution of male choosiness, with males achieving the same reproductive success through mating with females irrespective of their size.

Although males might obtain no fitness benefit through investing in larger females on the basis of fecundity, there might still be an advantage in doing so through superior egg quality, which often correlates with female size. In fishes, female size and egg size often correlate, and larger eggs have greater hatching success and produce larger larvae, which have reduced susceptibility to starvation, lower risk of predation, greater food capture abilities, and superior growth rates (Blaxter and Hempel 1963; Pepin 1991; Trippel 1998; Kamler 2008). We found no correlation between female size and egg volume, indeed egg volume showed little variation among females. In the case of bitterling, egg size may be more strictly conserved than in other fish species (Smith et al. 2004; Kitamura 2005). This is because egg size is under selection to match the width of mussel gill lamellae in which the eggs are deposited (Kitamura 2005; Reichard, Liu, et al. 2007; Reichard et al. 2010). Eggs that are too small or too large to fit inside the gill face the risk of ejection, and egg ejections are the chief cause of egg mortality during development (Kitamura 2005; Spence and Smith 2013). Thus, selection on egg size through coevolution with host mussels may constrain any variation in egg size linked to female size. Larger females may produce eggs of higher quality through superior provisioning (Brooks et al. 1997), though variation in this aspect of egg quality was not tested in this study.

The finding that males responded to the oviposition decisions of females is in agreement with a previous study where male leading rate was found to correspond with female mussel choice in the European bitterling (*Rhodeus amarus*) (Smith et al. 2002), which has a similar mating system to *R. ocellatus* (Pateman-Jones et al. 2011). Thus, this finding implies that females do not reference male behavior in measuring mussel quality and instead assess mussel quality independently. Females do attend to male cues in making spawning decisions. Mate choice decisions are based on olfactory cues that identify male genetic compatibility (sensu Neff and Pitcher 2005), such that females spawn preferentially with males with alleles complementary to their own. However, mussel quality, which represents a direct fitness benefit to females, is a better predictor of female oviposition than male genetic compatibility, which represents an indirect benefit (Agbali et al. 2012).

The failure of male *R. ocellatus* to discriminate among oviposition sites on the basis of quality as sites for egg incubation does not appear to disadvantage them given that females exercise strong oviposition preferences. However, this observation raises the question of why males inspect mussels, which they do repeatedly whether a female is present or not. Male *R. amarus* differentiate among mussels in response to the risk of sperm competition (Smith et al. 2003), with the presence of a rival's sperm representing the potential cue for assessing sperm competition risk. In both *R. amarus* and *R. ocellatus*, sperm competition is common (Smith et al. 2009; Pateman-Jones et al. 2011), and preoviposition ejaculation into mussels is a striking feature of the mating system, both by territorial mussel-guarding males and as a sneaking tactic (Kano 1996, 2000; Smith et al. 2002, 2004; Smith and Reichard 2005). Males regularly ejaculate into mussels, even in the absence of sperm competition, a behavior termed "topping-up" (sensu Parker 1998). Different mussel species may vary in the frequency with which they require

sperm to be replaced, which correlates with mussel filtration rate (Smith C, unpublished data). Thus, male and female *R. ocellatus* may use different cues in measuring mussel quality, which reflects differences in how selection acts on each sex during mating. In the case of females, selection acts most strongly on discriminating mussel traits related to offspring survival (Agbali et al. 2012). In males, mussels may be inspected to measure paternity prospects by assessing the level of sperm competition risk. In the present study, it was not clear why males devoted more time to inspecting *A. anatina*, rather than *U. pictorum*, because experimental fish had been routinely exposed to both mussels prior to experiments. Pilot studies using artificial mussels also implicated different cues in oviposition decisions by the sexes (Philips A, unpublished data), and future studies will explore the significance of alternative cues in mating decisions.

A number of substantial empirical studies now provide support for male mate choice (Edward and Chapman 2011). These demonstrate that male investment in mating, including the costs associated solely with courtship behavior and sperm production, can drive male mate choice (Werner and Lotem 2003). Thus, male choice can evolve in mating systems, such as polygynous and lek-mating systems, where male mate choice has hitherto not been considered. The challenge is to measure the costs associated with male expenditure on mating activities, the benefits of male choice, and the function of female ornaments in the context of mating system evolution.

In conclusion, this study demonstrated that male *R. ocellatus* do not invest more in courtship of females or aggressive behavior to rivals when presented with larger females. This result is explained by the absence of a correlation between female size and either clutch size or egg size. Despite inspecting mussels intensively, males did not discriminate between mussel species on the basis of their suitability as a site of incubation, though females did, and instead matched their preferences to those of females. These findings suggest males and females may use different cues in making oviposition decisions.

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