



Reproductive behaviour of female rosy bitterling *Rhodeus ocellatus* in response to a female-biased operational sex ratio

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Abstract

While the effect of Operational Sex Ratio (OSR) on reproductive behaviour of males has been studied extensively, little is known of the response of females facing a female-biased OSR. We investigated the effect of different OSRs on female reproductive behaviour using the rosy bitterling, *Rhodeus ocellatus*, a freshwater fish that lays its eggs inside the gills of living freshwater mussels. Three levels of OSR (male/female ratio 1:1, 1:3 and 1:5) were tested. We demonstrated that inspection of the mussel (spawning substrate) by individual females increased with increasingly female-biased OSR, but that the rate of following territorial male decreased. Aggression towards other females was not affected by the OSR. Interestingly, when a male bitterling led a non-dominant female towards the mussel, the dominant female would become aggressive to the male and chase the non-dominant female away. Aggression towards male followed a bell-shaped pattern and was highest at an OSR of 1:3. In both the female-biased OSRs examined, almost 50% of dominant females tended to chase away other females and defend the mussel, showing territoriality in a similar manner to males. These observations suggest that female reproductive behaviour is strongly affected by the OSR, and their reproductive tactics during courtship change from a passive role in courtship (following a male) to an active role in courtship (approaching a male), with presence of female territorial behaviour as the OSR becomes increasingly female-biased. This study provides strong evidence that a female-biased OSR has an important effect on female reproductive behaviour.

Keywords

alternative reproductive behaviour, female aggression, operational sex ratio, bitterling.

1. Introduction

Numerous studies have demonstrated that Operational Sex Ratio (OSR: the ratio of sexually active males to fertilisable females; Andersson, 1994) can strongly affect animal reproductive behaviour, since it indicates the level of expected intrasexual competition for mates or mating opportunities (Clutton-Brock & Parker, 1992; Taborsky, 1994; Kokko & Rankin, 2006). As the OSR deviates from equality, more intense mating competition is predicted, and the sex that is more abundant is predicted to become the competitor for access to mating partners (Kvarnemo & Ahnesjö, 1996, 2002). Therefore, individuals are expected to quickly modify their reproductive behaviours in response to changes in the local OSR.

Extensive theoretical and empirical research has focused on alternative male reproductive behaviours (reviewed by Taborsky, 1994). For example, the frequency of male aggression, sneaking and courtship disruption increased in Japanese rice fish, *Oryzias latipes*, as OSR became more male-biased (Grant et al., 1995). Male mosquitofish, *Gambusia affinis*, exhibited a correlative decrease in courtship and increase in male aggression as the proportion of males increased (Smith, 2007). In contrast, alternative female reproductive behaviours have attracted little attention. The few studies that have been completed suggest that female reproduction is indeed affected by the OSR. For example, aggressive female–female competition has been observed when the OSR was female-biased in both pipefish, *Syngnathus typhle* L., a species with sex-reversed mating roles (Vincent et al., 1994), and in Japanese rice fish, a species with conventional sex roles (Grant & Foam, 2002). Forsgren et al. (2004) showed that fierce male–male competition and intensive courtship behaviour was replaced by female–female competition and actively courting females in spotted gobies, *Gobiusculus flavescens*, during the transition from male-biased to female-biased OSR caused by demographic changes over a short breeding season.

Bitterling are a group of freshwater fish belonging to the subfamily Acheilognathinae (family Cyprinidae) that have a unique spawning symbiosis with freshwater mussels. During the spawning season, males develop bright nuptial colouration, defend territories around mussels and actively court females. Females follow males to their territory, with males undulating their body and directing females to mussels in their territory. Having followed a male, the females inspect the mussel exhalant siphon and if they

decide to oviposit, they lay their eggs inside the gills of the mussel by inserting long ovipositors into the mussel's gill cavity. Males then fertilize the eggs by releasing sperm into the inhalant siphon of the mussel such that water filtered by the mussel carries the sperm to the eggs. Bitterling display remarkable morphological, physiological and behavioural adaptations for using mussels as spawning sites and represent a valuable model in behavioural, population and evolutionary ecology (Smith et al., 2004).

Considerable research has been focused on alternative male reproductive behaviours influenced by male-biased OSR in bitterling (Candolin & Reynolds, 2002; Mills & Reynolds, 2003; Smith et al., 2003; Reichard et al., 2004); but the effect of variation in female-biased OSR on female reproductive behaviours has not been addressed. Here, we studied the effect of female-biased OSR on female mating behaviour using the rosy bitterling, *Rhodeus ocellatus*. We tested the prediction that female rosy bitterling are capable of changing their reproductive behaviour in response to changes in OSR.

2. Materials and methods

2.1. Field collection

A total of 36 mussels of the species *Unio douglasiae* were collected from Lake Poyang (Yangtze River Basin, 116°09'E, 28°23'N) using a long-handled dip net in 2009. These mussels were collected before the onset of the bitterling reproductive season to ensure that they did not contain any bitterling eggs. They were fed daily with a live algal suspension derived from an outdoor pool that had been seeded with *Chlorella vulgaris*.

A total of 36 male and 108 female rosy bitterling were collected from Bao'an lake (Yangtze River Basin, 114°23'E, 30°15'N, altitude 20 m) in Hubei province, P.R. China. The fish were transported to the Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan), and housed in stock aquaria with fresh water. The bitterling were then separated by sex, kept under a natural light regime and fed with frozen chironomid larvae and commercial fish flake fish food.

Little is known about the OSR of natural rosy bitterling populations during the breeding season, but it may be naturally female-biased as Zhang (2005) reported a male-to-female OSR of 0.64:1 in a small pond. In European bitterling, *Rhodeus amarus*, female bias in the population sex ratio

is common during the latter part of the reproductive season due to increased male mortality (Konečná & Reichard, 2011) and it is likely that a similar effect may also be expressed in some rosy bitterling populations. Importantly, while the population-level OSR of European bitterling is typically strongly male-biased (Smith et al., 2004), local conditions (e.g., low mussel availability, high variation in mussel or male quality) can result in the local OSR being female-biased (Reichard et al., 2004, 2008). It is very likely that local conditions are similar in the rosy bitterling.

In our experiments, the OSRs were set as 1:1, 1:3 and 1:5 (male/female) to investigate the effect over a broad range. There was always a single male in the tank and we only varied female density from 1 to 5. Therefore, when manipulating OSR, we also modified female density. For each OSR treatment, 12 replicates were conducted using different individuals. Experiments were conducted in a tank measuring 50 (length) \times 45 (width) \times 40 (depth) cm with a 5 cm deep sand substrate. Mean water temperature during the experiments was 22°C. All experiments were conducted in accordance with Chinese legal requirements.

2.2. *Observations of reproductive behaviours*

The breeding season of rosy bitterling lasts from April to September, with the peak during May to June (Shen, 2000). All our experiments were carried out by the same person (C.L.) from June to September 2009, i.e., at the time when a female-biased OSR was most likely to occur in the wild due to increased male mortality over the reproductive season (Konečná & Reichard, 2011). Before the start of experiments, a mussel was placed in a sand-filled plastic cup at the centre of the experimental tank and covered with an upturned perforated plastic cup to prevent females from spawning in them, though all bitterling were able to see and smell the mussel, thereby eliciting the full repertoire of reproductive behaviours (Spence et al., 2013). The appropriate number of female rosy bitterling with extended ovipositors (based on a random pre-determinate order) were then collected from the stock aquaria and gently released into the experimental tank and allowed to adapt to the new conditions. After introducing the females, a male was caught from the stock aquaria and added to the test tank, whereupon the perforated cup was removed and behaviour recording started. A 10-min video recording was made. The behaviours scored from the video footage were derived from Wiepkema (1961) and Smith et al. (2004), with frequency estimates made for the following behaviours:

1. Inspection: head down posture, females inspecting the mussel's exhalant siphon, assessing cues related to mussel suitability for offspring survival (Reichard et al., 2007).
2. Following: females following a leading male to a mussel as part of courtship activity. Male quivers his body and swims slowly towards the mussel, being followed by the female.
3. Aggression: females chasing the male or other females away (rapid swimming towards another fish, which invariably flees).
4. Territorial behaviour: a female defending a mussel and consistently chasing other females from the mussel; also includes attacking on the male if he courts another female (scored as presence or absence of territoriality, in addition to a quantitative measure of aggression).

After completion of video recording, fish were removed and measured (Standard Length, from tip of the snout to origin of the caudal fin) and stocked in a separate stock tank. No fish or mussel was re-used in the study.

2.3. Data analysis

All data were scored for individual females. Dominant females, identified by consistent aggression towards other females and to males, were individually followed on video footage and their behaviours were scored. In replicates with no dominant female, a randomly chosen female was followed over the observation period. We used General Linear Models (LMs) for normally distributed data and Generalised Linear Models (GLMs) for data with a Poisson distribution. Model fit was always checked for overdispersion, and a quasi-Poisson rather than Poisson error distribution was used when overdispersion was detected. Note that we only compared two treatment groups for female aggression to other females as this behaviour cannot be recorded with an OSR of 1:1, i.e., with a single female in the tank. All statistical analyses were performed in R environment, version 3.0.0 (R Development Core Team, 2007).

3. Results

3.1. Inspection and following behaviour

Females performed inspection behaviour at all OSRs and its rate significantly increased at female-biased OSRs (LM: $F_{2,33} = 3.62$, $p = 0.038$; Figure 1a).

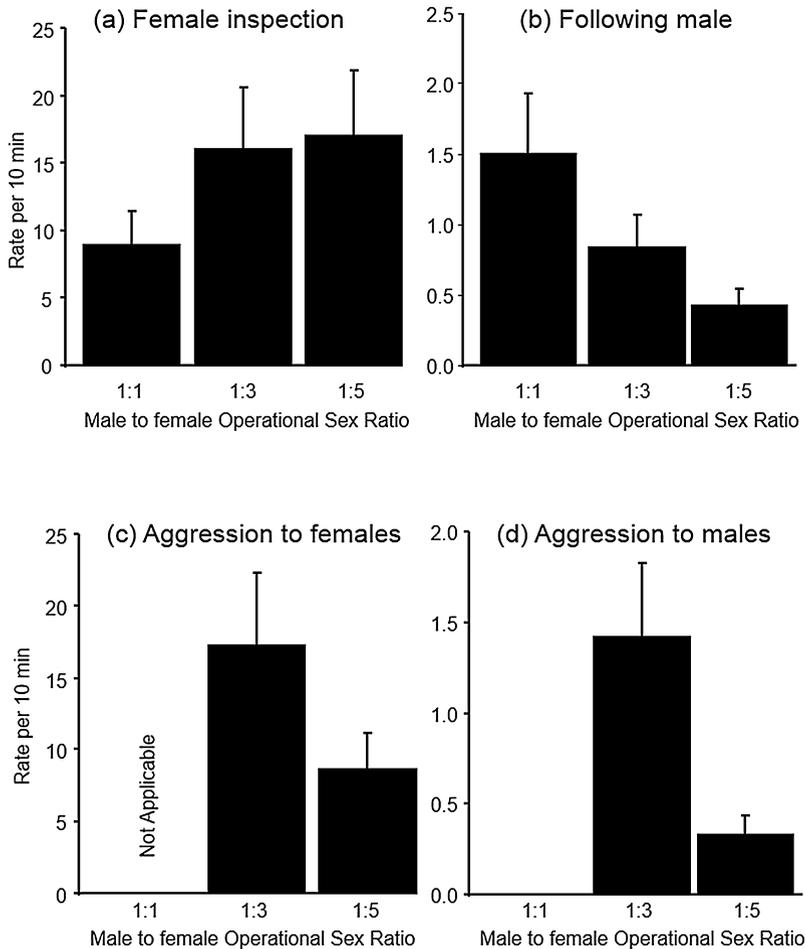


Figure 1. Female reproductive behaviours in different OSRs. (a) Mean (\pm SE) frequency of mussel inspection; (b) mean (\pm SE) frequency of following a male; (c) mean (\pm SE) frequency of aggression at females; (d) mean (\pm SE) frequency of aggression at the male. All values are rates per 10 min.

There were no pair-wise differences in inspection behaviour between the 1:3 and 1:5 OSR (Tukey Post Hoc Tests, $p > 0.05$). The frequency of following behaviour for individual females decreased with increasing OSR (GLM with Poisson distribution: $df = 2, 33, \chi^2 = 7.94, p = 0.019$; Figure 1b).

3.2. Female aggression towards other females

By default, there was no female aggression towards other females at an OSR of 1:1. Females were aggressive to other females at female-biased sex ratios, but there was no significant difference in female aggression between OSRs of 1:3 and 1:5 (GLM with quasi-Poisson distribution, $F_{1,22} = 1.57$, $p = 0.223$, Figure 1c).

3.3. Female aggression towards male

An interesting phenomenon, when the dominant female chased away other females led by the male towards the mussel her aggression towards the male also escalated. Female aggression exhibited a bell-shaped pattern, with a steep increase in aggression towards male at an OSR of 1:3, followed by a decrease at an OSR of 1:5 (GLM with Poisson distribution: $df = 2, 33$, $\chi^2 = 25.69$, $p < 0.001$; Figure 1d).

3.4. Female territoriality

At an OSR of 1:1, females showed no territorial behaviour, but female territorial behaviour was recorded at female-biased OSRs (GLM with quasi-binomial distribution: $F_{2,33} = 7.82$, $p = 0.002$, Figure 2). It is interesting to note that females sometimes performed a trial spawning behaviour, termed *skimming* (Smith et al., 2004). They quickly swept forward and down, but did not push their ovipositors into the mussel's exhalant siphon. When eggs

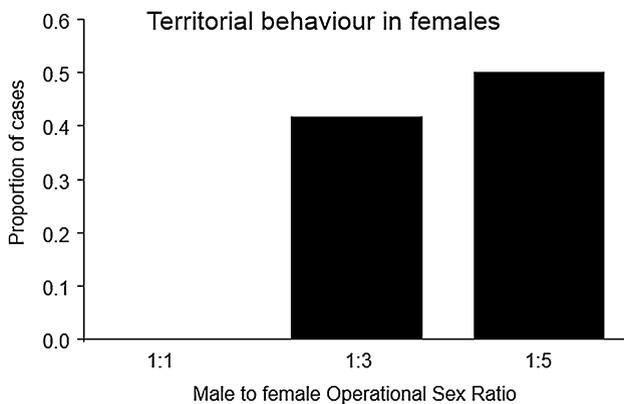


Figure 2. Percentage of cases when one female became territorial fish from a total of 12 replicates for each OSR treatment. Note that the female cannot be scored as territorial in OSR 1:1.

were released, they did not reach the mussel's gills and were eaten by the fish immediately. This behaviour occurred too rarely to test the rate of its occurrence statistically (in 3 females at OSR 1:1, 2 females at OSR 1:3, and 3 females at OSR 1:5).

4. Discussion

4.1. Sex ratio dynamics

In wild populations, sex ratios (including OSR) display many dynamic features (Pettersson et al., 2004). This means that local sex ratios, and their consequences for sexual selection, also need to be seen from a dynamic perspective. In two-spotted gobies, *G. flavescens*, for example, a change has been observed in OSR from heavily male-biased early in the season to heavily female-biased at the end of the short breeding season (Forsgren et al., 2004). In the mating system of the longnose filefish, *Oxymonacanthus longirostris*, an unbiased adult sex ratio at the beginning of the breeding season became slightly female-biased toward the end due to a decline in the number of males (Kokita & Nakazono, 1998). This decline in male abundance is most likely due to higher male mortality during the breeding season (Konečná & Reichard, 2011), possibly caused by exhaustion resulting from parental care or male–male competition, a higher susceptibility to parasites and diseases due to the immunosuppressive effect of testosterone (Folstad & Karter, 1992), or increased predation risk (Marconato et al., 1993; Macías Garcia et al., 1998; Lindström, 2001). One study suggests that sex ratio during the breeding season may be naturally female-biased in wild rosy bitterling populations (Zhang, 2005), though the causes of this bias are not yet known. Notably, local conditions, such as low mussel availability and/or high variation in mussel or male quality, can often result in female-biased local OSRs where several females co-occur in a single male territory simultaneously (Reichard et al., 2008). This situation creates a further dynamic aspect to the OSR, with direct implications for individual reproductive behaviour (Reichard et al., 2004).

4.2. Effect of the OSR on female reproductive behaviours

Our experiments indicated that female rosy bitterling reproductive behaviour was affected by the OSR. Frequency of inspection significantly increased as the OSR became increasingly female-biased (Figure 1a). However, female

aggression towards other females did not significantly increase with a greater female bias in the OSR (Figure 1c). This finding is not fully consistent with research in some other species. For example, in *O. latipes*, the frequency of female–female aggression increased as the ratio of female to male increased from 0.66 to 1.9, though it decreased again slightly at OSRs between 1.9 and 3 (Grant & Foam, 2002). In the peacock blenny, *Salaria pavo*, females attacked other females rather than guarding males and floater males when nest sites were scarce and courtship episode frequency increased with frequency of agonistic interactions between females in each nest (Almada et al., 1995). In general, when the resources necessary for successful breeding are heavily clustered, reproductive competition between females may become frequent or intense. However, in rosy bitterling, the intensity of female–female aggression is not significantly influenced by the OSR, at least over the range of values we tested. This may indicate that female–female aggression is an ineffective strategy for obtaining more mating opportunities when rosy bitterling OSR becomes increasingly female-biased. Alternatively, aggression towards other females is already high at an OSR of 1:3 and females may not possess the capacity to elevate their aggression further.

Following behaviour decreased with the increasing number of competing females, possibly as a trade off with increased inspection of the mussel and female aggression. Indeed, at an OSR of 1:3 and 1:5, females tended to chase other females away and guard the mussel as territorial females in the same manner that males typically do. As competition for both males and resources is high at a female-biased OSR, females tend not to wait for males to perform courtship and leading behaviour and instead monopolise oviposition sites, thereby preventing other females from mating (Emlen & Oring, 1977). These observations suggest that female reproductive tactics may change from a passive role to active courting and territorial defence when the OSR becomes increasingly female-biased.

4.3. Female aggression towards a male

Aggression usually occurs among males due to differential rates of reproduction between males and females (Clutton-Brock & Parker, 1992), but it may also occur among females in “sex-role-reversed” species (Colwell & Oring, 1988). Adaptive female responses may also be plausible for situations when the OSR is strongly female-biased, as we report in the present study. Female aggression towards males sometimes occurs in socially monogamous mating systems, or in pairs with biparental care (Andersson, 1994). However,

female aggression towards a male in the mating context in taxa with conventional sex roles and no parental care is unusual. In the present study, such aggression was observed when male bitterling led a non-dominant female towards the mussel; the territorial female attacked the male immediately after chasing the rival female away. While female aggression towards a male may be a costly behaviour, the benefits of interrupting the courtship with a rival female during territorial defence may lead to a net maximization of her own fitness. Such behaviour was, however, rare and aggression to males occurred approximately 10 times less than aggression to other females. In the present study, the incidence of female aggression is consistent with the predictions of Emlen & Oring (1977), with aggression peaking at intermediate OSRs and declining in the context of the economic defensibility of resources (Emlen & Oring, 1977; Reichard et al., 2004; Klug et al., 2010; Weir et al., 2011).

4.4. Operational sex ratio and alternative reproductive behaviour

Our previous experiments have shown that reproductive behaviours of male rosy bitterling were strongly affected by the OSR (Liao et al., 2013), with increased aggression and pre-oviposition ejaculation and decreased leading as two alternative responses to increasingly male-biased OSR. In the European bitterling, males also adopt alternative behaviours in response to an increased male-biased OSR. Candolin & Reynolds (2002) and Řežucha et al. (2012) have found that increased ejaculation rate and increased aggression were alternative responses to the increased risk of sperm competition. In the present study, when female rivals were absent, females inspected mussels and followed males during courtship. At an OSR of 1:3, however, females became highly aggressive toward both rival females and the male, while the rate of following decreased. When the number of rival females was increased further, females allocated most energy to territorial behaviour, which involved defending the mussel. Therefore, both male and female bitterling, therefore, are sensitive to the changes in OSR and alter their reproductive tactics to maximize the fitness benefits.

Whereas OSR can affect breeding system structure either independently or concomitantly with density (Kodric-Brown, 1988), density generally affects the magnitude of OSR effect and not the directional change in reproductive behaviour (Arnqvist, 1992; Alonso-Pimentel & Papaj, 1996; Weir et al., 2011). In the rosy bitterling, experimental studies have confirmed the role of high male density on elevating male aggression (Kanoh, 2000). In our experiment, we inadvertently manipulated female density along with changes

in OSR. We feel, however, that such an approach was the only one reasonable logistically, given the mating system of the bitterling, as any increase in the number of males would introduce major changes to male behaviour (Liao et al., 2013), and any effect on female behaviour would be masked. Further, our interpretation is biologically plausible and other evidence, such as the absence of female aggression towards other females and especially towards a male from non-experimental situations with a female-biased OSR and different population densities (i.e., in breeding tanks or natural observations), suggest that the role of the OSR is primary.

To conclude, according to OSR theory, when faced with a bias in OSR and reproductive competition, both males and females are predicted to alter their reproductive tactics to maximize their fitness outcomes. While extensive empirical data have demonstrated that males may respond to a male-biased OSR, here we demonstrate that females also respond to a female-biased OSR by changing reproductive tactics from a passive role (following a male who possesses a territory and actively courts females) to an active role (aggressively defending a resource from other females and restricting their mating opportunities and actively approaching a male). These findings have implications for our understanding of flexibility in animal mating systems in response to variation in the availability of mates.

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