Operational sex ratio affects nest tending and aggression in male flagfish *Jordanella floridae* Goode & Bean

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Operational sex ratio (OSR, the ratio of sexually active males to fertilizable females at a given time and location) affected male behaviour in the flagfish *Jordanella floridae*. When OSR was male biased, males spent (1) more time at their nests and (2) more time fanning prior to receiving eggs. Pre-mating fanning has previously been correlated with male mating success and is hypothesized to be used in female choice in this population. Thus, these results suggest that on average, male flagfish invest more time in behaviour associated with female choice when there are relatively more male competitors. The OSR also affected the frequency of male aggression, and specifically male aggression towards females was more frequent at female-biased OSR treatments. The observed patterns were dependent upon the direction of OSR bias (i.e. unbiased, male biased and female biased), and in some cases the intensity of the OSR bias affected the patterns of behaviour. These findings suggest that experimentally detecting effects of OSR is sensitive to the specific OSR values considered, and highlight the importance of considering a range of OSR values in future studies.

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Key words: *Jordanella floridae*; operational sex ratio; parental care; potential rate of reproduction; sexual selection.

INTRODUCTION

The operational sex ratio (OSR), the ratio of sexually active males to fertilizable females in a population at a given time (Emlen & Oring, 1977), is frequently used to predict the direction and intensity of sexual selection (i.e. intra-sexual variation in mating success) and more generally, has been used to explain and predict differences in mating systems, reproductive behaviour and sexual dimorphism (Andersson, 1994; Kvarnemo & Ahnesjö, 1996; Forsgren *et al.*, 2004). In most systems, OSR is thought to be male biased because females often have a lower potential rate of reproduction, (i.e. offspring...
production per time unit for each sex when mate availability is unconstrained, PRR), than males (Clutton-Brock & Parker, 1992; Ahnesjö et al., 2001; Kvarnemo & Ahnesjö, 2002). Such biases in OSR are expected to lead to intra-sexual competition for mates, and as the limiting sex (typically females) becomes scarcer, the intensity of sexual selection and mate competition is expected to increase (Emlen & Oring, 1977; Clutton-Brock & Parker, 1992; Ahnesjö et al., 2001).

Several studies have evaluated the effect of OSR on mating behaviour. Consistent with predictions suggesting that OSR should affect mating behaviour (Kvarnemo & Ahnesjö, 1996, 2002), male–male aggression increased at male-biased OSRs in the sand goby Pomatoschistus minutus (Pallas) (Kvarnemo et al., 1995), sockeye salmon Oncorhynchus nerka (Walbaum) (Quinn et al., 1996), the guppy Poecilia reticulata Peters (Jirotkul, 1999a) and the two-spotted goby Gobiusculus flavescens (Fabricius) (Forsgren et al., 2004). In the Japanese medaka Oryzias latipes (Temminck & Schlegel), OSR influenced the intensity of male–male aggression (Grant et al., 1995). Likewise, more female–female competition was observed when OSR was female biased in the pipefish Syngnathus typhle L. (Vincent et al., 1994), the Japanese medaka (Grant & Foam, 2002) and the two-spotted goby (Forsgren et al., 2004). Male choosiness in females increased as the proportion of males in the population increased in guppies (Jirotkul, 1999a), and when female PRR was lower than male PRR in St Peter’s fish Sarotherodon galilaeus (L.) (Balshine-Earn, 1996). Similarly, male mate-choice was more prevalent when OSR was female biased in the pipefish (Berglund, 1994). In some systems, however, changes in OSR were either unrelated to changes in behaviour associated with mate competition, or the direction of OSR bias and mating competition were negatively correlated (Kvarnemo & Ahnesjö, 2002). For example, increasing OSR in the beaugregory damselfish Stegastes leucosticus (Müller & Troschel) did not affect male mating success (Cleveland et al., 2002), and Denoël et al. (2005) found no effect of OSR bias on courtship behaviour or responsiveness of females towards males in the Alpine newt Triturus alpestris (Laurenti). Likewise, variance in male mating success decreased at more male-biased OSRs in the European bitterling Rhodeus sericeus (Pallas) (Mills & Reynolds, 2003) in comparison to unbiased or female-biased OSRs, because males exhibited alternative mating behaviour when male density was relatively high.

While previous work suggests that both the direction and the intensity of OSR bias are likely to affect mating behaviour (Kvarnemo & Ahnesjö, 1996; Bessa-Gomes et al., 2003; Forsgren et al., 2004), most experimental work on the topic has focused on a narrow range of OSRs and typically only considers effects of the direction of OSR bias. Indeed, many studies consider at most three OSRs (e.g. unbiased, male biased or female biased; Höglund, 1989; Kvarnemo et al., 1995; Cleveland et al., 2002; Mills & Reynolds, 2003; Denoël et al., 2005; Head & Brooks, 2006) and studies considering a range of OSRs are rare (Grant et al., 2000; Jirotkul, 2000; Forsgren et al., 2004). To explore importance of the direction and intensity of OSR bias on mating behaviour, the effects of five OSR treatments on male behaviour were evaluated in the flagfish Jordanella floridana Goode & Bean, a species in which males alone provide parental care of eggs. In particular, pre-mating fanning and male aggression
were focused on, as these behaviours have previously been positively correlated with mating success in the flagfish (St Mary et al., 2001; Hale, 2006), and thus, it was hypothesized that these behaviours would be affected by OSR.

MATERIALS AND METHODS

STUDY SPECIES

Flagfish are primarily freshwater fish found in the shallow waters of Florida, the coast of the Gulf of Mexico and the Yucatan (Mertz & Barlow, 1966). Flagfish live c. 1 year, and they breed from late March to September (Mertz & Barlow, 1966). During the breeding season, males establish and defend nesting territories. In the wild, flagfish nests are typically a site within a male’s territory, such as a plant or patch of vegetation, and experimental work suggests that males can nest and care for eggs continuously throughout the breeding season (Klug & St Mary, 2005). Brood cycling is absent in the flagfish, i.e. nest-holding males continuously accept eggs from females while providing care (Mertz & Barlow, 1966), and nest size does not appear to limit the number of eggs a male can receive, as nests have never been observed to be full with eggs (Klug & St Mary, 2005; Klug et al., 2005). Females are continuous breeders, they can spawn repeatedly on a daily basis and mate with multiple males throughout the breeding season (Mertz & Barlow, 1966).

Male aggression (e.g. chasing of males and females) is frequent among nesting males and has previously been correlated with mating success in this species (St Mary et al., 2001). As mentioned previously, males alone provide parental care of eggs, including nest guarding, cleaning and fanning. Males also fan their nests prior to receiving eggs; such pre-mating fanning is thought to play an important role in female choice and has previously been correlated with male mating success in flagfish (St Mary et al., 2001).

EXPERIMENTAL DESIGN

The experiment was conducted in 2005 in Gainesville, FL, U.S.A. during the flagfish breeding season (June to August). All fish were collected from the Otter Creek – Waccasassa River drainage in north-west central Florida and housed in large 150 l holding tanks prior to the start of the experiment. Density in the holding tanks was maintained at c. 0.5 fish l⁻¹, and all fish were housed in a holding tank for at least 10 days prior to use in the experiment. Following the experiment, all fish were transferred to separate holding tanks and were subsequently used in other behavioural experiments unrelated to the work described here. All experimental tanks (500 × 260 × 300 mm) were equipped with an airstone, artificial plastic plants and one spawning mat (a 0.01 m² ceramic tile with a green acrylic felt carpet glued on top) per male in the tank. Nests were c. 260 mm apart in each treatment and were large enough to hold eggs from all females in a tank, and nests were never observed to become full with eggs. All tanks were maintained at c. 28°C and experienced a 14 L:10 D cycle. The fish were fed a diet of algae tablets and frozen brine shrimp Artemia sp. ad libitum throughout the experiment.

Empirically, OSR was defined as total number of males prepared to mate divided by the total number of males and females, i.e. the total number of fish, prepared to mate at a given time and place (Kvarnemo & Ahnesjö, 1996). As both males and females continue to remain receptive after spawning, the OSR within a given experimental tank did not change as spawning occurred. There were six experimental treatments: (1) one male, one female, OSR = 0.5 (n = 10), (2) two males, two females, OSR = 0.5 (n = 9), (3) two males, one female, OSR = 0.67 (n = 9), (4) four males, one female, OSR = 0.8 (n = 10), (5) one male, two females, OSR = 0.33 (n = 10) and (6) one male, four females, OSR = 0.2 (n = 10). To ensure that all fish were reproductive, only males with reproductive colouration and gravid females were used. After assigning a tank to one of
the six treatments, the appropriate number of males and females were added to the tank on day 0. Males were placed in the main compartment of the tank and allowed 3 days to establish nesting territories prior to behavioural observations. Females were placed in individual mesh boxes within the tank (which allowed visual and chemical, but not physical contact, with males) for an acclimation period of 3 days. Females were then released into the main compartment of the tank (with the males) on day 3. Each tank was filmed for 10 min using a C-VHS camcorder between 1100 and 1400 hours days on 4 and 5. Additionally, the presence and number of eggs on the spawning mats was recorded daily until day 7. Eggs were found in only eight nests during the experiment. Specifically, eggs were found on day 6 in seven tanks and on the evening of day 4 in one tank, and in all cases, eggs were found in one nest per tank. For the male that received eggs on the evening of day 4, no eggs remained on day 5, and thus males never had eggs in their nests during either of the filming dates. On day 7, each experimental replicate was ended and females and males were weighed (M) and their standard length (Ls) was measured.

BEHAVIOURAL OBSERVATIONS

Male behaviour was quantified from the videotapes using the focal sampling software programme JWatcher 0.9 (Blumstein et al., 2000). It was impossible to reliably keep track of individual fish within a tank, and thus, for all behavioural observations, one male was randomly selected in each tank and only his behaviour was quantified. Randomly selecting one male presumably allowed for the assessment of average male behaviour and avoided any biases that might have arisen from selecting only highly active males. It is important to note, however, that OSR might affect within-tank variation in behaviour, or it might affect certain males (e.g. highly aggressive or dominant males) differently than other males, and such effects were not assessed in this experiment. For each behavioural observation, the proportion of time the male spent at the nest, the rate of nest fanning (defined as angling the body downward over the nest and oscillating the body back and forth; St Mary et al., 2001), the average proportion of time males spent fanning, the rate of male chases directed at females (defined as an accelerated movement towards a female), and the rate of male chases directed at other males (defined as an accelerated movement towards another male) were quantified. The proportion of time and rate of engaging in each of the behaviours were averaged between the two filming dates for each tank.

STATISTICAL ANALYSES

To determine and control for potential effects of absolute density (over the narrow range of densities considered in this experiment), ANOVA was used and comparisons were made between (1) only the two control treatments in which OSR was unbiased (treatment 1, low density, and treatment 2, high density) and (2) all treatments by considering density a categorical variable (low density = two to three fish, high density = four to five fish). Because there was never an effect of density on any behaviours quantified, density was not included as a factor in subsequent analyses.

The effects of OSR on male reproductive behaviour were analysed in two ways. First, to consider the effect of both the direction and the intensity of OSR bias, the effect of the OSR value (0/2, 0·33, 0·5, 0·67 and 0·8) was evaluated on all behaviours using ANOVA and logistic regression. Second, the analyses were performed considering only the direction of the OSR bias (unbiased, female biased or male biased) on male behaviour. Pooling the data increased statistical power in some cases, and it also allowed for the consideration of the more general effect of the direction of OSR bias. In both cases, subsequent post-hoc Tukey HSD analyses were performed to assess differences between group means when main effects were significant at the $P < 0·05$ level. Additionally, because behaviour can be thought of as involving a two-step decision process (where the first decision is whether or not to engage in the behaviour and the second decision...
involves deciding how frequently to display the behaviour), the frequency of behaviours were analysed both including and excluding males that never performed a particular behaviour (e.g. fanning, chasing males and chasing females). For all analyses in which the mean rate of chasing males was considered, it was necessary to exclude treatments in which only one male was present, i.e. treatments 1, 5 and 6. All statistical analyses were performed using SPSS 12.0 software (SPSS Inc., Chicago, IL, U.S.A.). All data were tested for normality and when assumptions of normality could not be met, a non-parametric test was used.

RESULTS

MALE AND FEMALE SIZE AND DENSITY

There were no significant differences between the OSR treatments in male mass (ANOVA, $F_{4,55}, P > 0.05$) or $L_S$ ($F_{4,55}, P > 0.05$), or in female $M$ ($F_{4,54}, P > 0.05$) or $L_S$ ($F_{4,57}, P > 0.05$). Mean ± S.E. male $M$ and $L_S$ were 2.16 ± 0.14 g and 36.6 ± 1.0 mm ($n = 106$). Mean ± S.E. female $M$ and $L_S$ were 0.98 ± 0.05 g and 29.4 ± 0.6 mm ($n = 107$). There was no effect of density on any of the male behaviours quantified when the two control treatments were considered (treatments 1 and 2, Kruskal–Wallis test, d.f. = 1, $P > 0.05$ in all cases) or when density was treated as a categorical variable (i.e. high density, four to five fish or low density, two to three fish; ANOVA, d.f. = 1, $P > 0.05$ in all cases).

PRE-MATING NEST TENDING

Proportion of time spent at the nest

While males tended to spend a greater proportion of time at the nest when OSR was male biased [Fig. 1(a)], the proportion of time males spent at the nest was not significantly different among the OSR treatments [ANOVA, OSR effect, $F_{4,53}$ and $P > 0.05$; Fig. 1(a)]. When the data were pooled and only the direction of OSR bias (unbiased, female biased or male biased) was considered (which increased statistical power), however, there was a significant effect of the direction of OSR bias and specifically, males spent more time at the nest when the treatments were male-biased [ANOVA, $F_{2,55}, P < 0.05$; Fig. 1(b)].

Fanning

Of the focal males, 33% exhibited at least one bout of fanning, and there was no effect of OSR on whether males exhibited fanning (logistic regression, OSR effect, d.f. = 4, $\chi^2, P > 0.05$; direction of sex-ratio bias effect, d.f. = 2, $\chi^2, P > 0.05$). When all males, i.e. both males that fanned at least once and males that never fanned, were considered, the mean rate of fanning was not significantly affected by the OSR value (Kruskal–Wallis test, d.f. = 4, $\chi^2, P > 0.05$) or the direction of OSR bias (d.f. = 2, $\chi^2, P > 0.05$). Similarly, the proportion of time males spent fanning was not significantly affected by OSR (Kruskal–Wallis test, d.f. = 4, $\chi^2, P > 0.05$) or the direction of the OSR bias (d.f. = 4, $\chi^2, P > 0.05$).
When males that fanned at least once were considered (and males that never fanned were excluded), there was no effect of OSR or the direction of the sex-ratio bias on the rate of fanning (ANOVA, OSR effect, $F_{4,14}, P > 0.05$; direction of sex-ratio bias effect, $F_{2,16}, P > 0.05$). While the proportion of time spent fanning was greatest at the male-biased OSR treatments [Fig. 2(a)], the effect of OSR on the proportion of time spent fanning was not significant (ANOVA, OSR effect, $F_{4,14}, P > 0.05$). When the direction of sex ratio bias was considered (by pooling the data), the proportion of time males fanned increased significantly when sex ratio was male-biased (ANOVA, direction of sex ratio bias effect, $F_{2,16}, P < 0.05$) [Fig. 2 (b)].

**AGGRESSIVE BEHAVIOUR**

*Chases directed at females*

Eighty-eight per cent of focal males chased females on at least one occasion. There were no effects of the OSR value or the direction of sex-ratio bias on
whether or not males exhibited chases directed at females (logistic regression, OSR effect, d.f. = 4, $\chi^2$, $P > 0.05$; direction of sex ratio bias effect, d.f. = 2, $\chi^2$, $P > 0.05$). The OSR, however, significantly affected the rate at which males chased females (ANOVA, $F_{4,53}$, $P < 0.01$). Specifically, males chased females more at unbiased and moderately female-biased OSRs [Fig. 3(a)]. Likewise, when only the effects of the direction of OSR bias were considered, males chased females significantly more at unbiased and female-biased OSRs [ANOVA, $F_{2,53}$, $P < 0.001$; Fig. 3(a)]. These findings were qualitatively identical when males that never chased females were excluded (OSR effect, $F_{4,46}$, $P < 0.001$; direction of sex-ratio bias effect, $F_{2,48}$, $P < 0.001$). In separate analyses, the effect of OSR on the per-female chase rate, i.e. the mean rate of chases directed at females divided by the number of females present, was evaluated. Again, the frequency of chases directed at females was significantly greater at unbiased and moderately female-biased OSRs (ANOVA, OSR effect: $F_{4,53}$,
Because males could only chase other males when there were at least two males present, this behaviour was only observed in three treatments, and thus, no comparisons of male chasing were made at female-biased OSRs. Eighty-nine per cent of males chased another male at least once. There was no effect of OSR (logistic regression, d.f. = 2, $\chi^2, P > 0.05$) or the direction of the sex-ratio bias (d.f. = 1, $\chi^2, P > 0.05$) on whether or not males exhibited male-directed chases. Additionally, there was no significant effect of OSR ($F_{2,25}, P > 0.05$) or the direction of the sex ratio bias ($F_{1,26}, P > 0.05$) on the rate of chases directed at other males. This trend was the same when males that never chased another male were excluded (OSR effect, $F_{2,22}, P > 0.05$; direction of sex ratio bias effect, $F_{1,23}, P > 0.05$).

Fig. 3. The effect of (a) operational sex ratio (OSR) and (b) the direction of the OSR bias on the rate of male flagfish chases directed at females. Values are means ± s.e. [the number of replicates per treatment (n) is indicated above bars]. Post-hoc analyses were performed when main effects were significant at the $P < 0.05$ level. Significance ($P < 0.05$) in post-hoc analyses is indicated by a and b.

$P < 0.05$; direction of sex-ratio bias effect, $F_{2,48}, P < 0.05$), suggesting that the increased rate of chasing at unbiased and female-biased OSRs is not an effect of increased female-encounter rate.

Chases directed at males

Because males could only chase other males when there were at least two males present, this behaviour was only observed in three treatments, and thus, no comparisons of male chasing were made at female-biased OSRs. Eighty-nine per cent of males chased another male at least once. There was no effect of OSR (logistic regression, d.f. = 2, $\chi^2, P > 0.05$) or the direction of the sex-ratio bias (d.f. = 1, $\chi^2, P > 0.05$) on whether or not males exhibited male-directed chases. Additionally, there was no significant effect of OSR ($F_{2,25}, P > 0.05$) or the direction of the sex ratio bias ($F_{1,26}, P > 0.05$) on the rate of chases directed at other males. This trend was the same when males that never chased another male were excluded (OSR effect, $F_{2,22}, P > 0.05$; direction of sex ratio bias effect, $F_{1,23}, P > 0.05$).
DISCUSSION

Operational sex ratio affected male pre-mating fanning and pre-mating aggression towards females in flagfish. Males spent more time at the nest and spent a greater proportion of time fanning when OSR was male biased, and specifically, males spent the greatest proportion of time fanning when OSR was highly male biased (Fig. 2). Pre-mating fanning in the flagfish has previously been correlated with subsequent mating success and is thought to be associated with female choice in this population (St Mary et al., 2001; Bonnevier et al., 2003). Thus, the findings of the present study suggest that, on average, males invest more time in behaviour associated with female choice when there are relatively more same-sex competitors present. This finding is in part contrary to work in some other species. For example, duration of male courtship was shorter and males displayed less at male-biased OSRs in one guppy population (Jirotkul, 1999a), there was no difference between male- and female-biased OSRs in the per male rate of displaying in another guppy population (Head & Brooks, 2006), and courtship decreased at male-biased OSRs in the Japanese medaka (Grant & Foam, 2002). Despite such decreases in courtship behaviour at male-biased OSRs, however, Jirotkul (1999a) found that female preference for males with orange colouration increased as OSR increased, suggesting that female choice remains important at male-biased OSRs in guppies. Alternatively, it is possible that pre-mating fanning in the flagfish also functions as a display to other males. Indeed, more work is needed to understand the specific fitness consequences of pre-mating fanning at different OSRs, and directly assess the effect of OSR on female preferences.

The rate of male chases directed at females increased at female-biased or unbiased OSRs, and this increased chasing does not appear to simply be an artefact of increased female encounter rate. In some other systems, male aggression has been found to increase with male-biased OSRs (Kvarnemo et al., 1995; Quinn et al., 1996; Jirotkul, 1999a; Head & Brooks, 2006). In the flagfish, male aggression has previously been correlated with mating success (St Mary et al., 2001), but it is unclear if this behaviour plays a role in sexual coercion, male–male competition, or female choice. Alternatively, increased chasing might suggest that males are becoming choosier at unbiased and female-biased OSRs. Regardless, OSR affected the rate of male chases directed at females in this study, and in the future it will be important to directly quantify the long-term fitness consequences of varying mating behaviours, i.e. aggression, pre-mating fanning, over a range of OSRs.

The qualitative patterns observed in this experiment were in general similar regardless of whether the specific OSR value, which reflects both the direction and intensity of the bias, or simply the direction of OSR bias was considered. Considering a range of OSRs, however, was more informative than considering only three OSR categories (unbiased, male biased and female biased). Indeed, some of the patterns would have been missed if only the direction of OSR bias (or a narrower range of OSRs) had been considered. For example, males tended to chase females more at unbiased and slightly female-biased OSRs, in comparison to male-biased or highly female-biased OSRs. In this case, the decrease in chasing at highly female-biased OSRs might have been missed if
a narrow range of OSRs had been considered. Likewise, for the proportion of time males spent fanning (Fig. 2), the observed effect sizes for comparisons between unbiased and moderately-biased OSRs, i.e. OSRs of 0.33, 0.5 and 0.67, were relatively small, and thus, it is possible that effects of OSR on male fanning would not have been detected if only a narrow range of OSRs had been considered. Thus, it is likely that detecting effects of OSR experimentally is sensitive to the specific OSR values considered. This finding might help explain lack of effects of OSR observed in other studies, and is consistent with previous empirical work suggesting that effects of OSR on mating behaviour are sensitive to the specific OSR values considered (Forsgren et al., 2004).

Finally, density was not found to influence any of the behaviours quantified in the experiment, suggesting that any differences in male behaviour could be attributed to manipulation of the OSR in this study. It is important to note that the range of densities examined in this study was very limited. While the patterns in the experiment do not appear to be due to absolute density, actually quantifying the effects of density per se on flagfish behaviour would necessitate the consideration of a wider range of densities. In general, additional work is needed to assess the importance of absolute density on sexual selection and associated behavioural strategies. While recent theoretical work suggests that sexual selection might be density dependent (Kokko & Rankin, 2006), this idea has rarely been considered empirically (Jirotkul, 1999b).

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