The effect of crowding and density on male mating behaviour in the rose bitterling (*Rhodeus ocellatus*)

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Summary

Female density and resource availability are two key variables that shape mating systems. Theory predicts that reproductive skew will amplify with increased male density and decreasing availability of resources, though limited empirical evidence suggests that this may not always be the case. Here we tested mean crowding, defined as the number of males per unit of resource, and density per se, defined as the number of individuals present per unit area, to investigate their effect on the mating system of *Rhodeus ocellatus*, a fish with a promiscuous, resource-based mating system. Males were exposed to combinations of high and low levels of crowding and density, while the operational sex ratio was held constant. High levels of crowding significantly affected the proportion of mussel spawning sites defended by males and the proportion of mussels into which sperm was released. In contrast to theoretical predictions, neither density nor crowding influenced overall male aggressive behaviours. Density, but not crowding, had a significant effect on male courtship rate, which arose as a possible trade-off between intra-sexual competition and inter-sexual behaviour. We discuss the results in the context of mating system evolution.

Keywords: density-dependence, mating system, sexual selection, sperm competition, territoriality.

Introduction

Differences in potential reproductive rate, resulting from anisogamy, have driven the evolution of sex role differentiation, whereby the sex limited by

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mate availability, almost always males, competes for fertilisations (Clutton-Brock & Parker, 1992). Thus, males are subject to intra-sexual selection for characteristics that enhance their competitive ability, and inter-sexual selection for traits that enhance their attractiveness to females (Andersson, 1994).

Kokko & Rankin (2006) argued the case for an impact of population density on the strength of sexual selection. Using a modelling approach they examined how density-dependent effects might operate on sexual selection, and how density might thereby impinge on population dynamics, proposing that sexual selection could operate as a self-limiting process mediated by density. For example, at low population density males may experience little competition, while at higher densities reproductive skew might be greater as males with greater resource holding potential monopolise matings to the exclusion of inferior males. However, while theoretical studies may predict that reproductive skew will increase asymptotically with density, empirical evidence suggests that this may not always be the case, for example because of switches in male behaviour (Jirotkul, 1999), the breakdown of resource defence (McLain, 1992; Reichard et al., 2004a,b), variation in resource availability (Wootton et al., 1995), changes in sex ratio (Pröhl, 2002), or because the effects of density may be weak (Head et al., 2008). The impact of alternative male mating tactics in particular can have a marked effect on reducing variance in male mating success (Reichard et al., 2004a,b; Taborsky, 2008).

Economic defendability theory predicts that the intensity of competition for a resource will correlate positively with population density and negatively with resource availability (Brown, 1964). However, below a certain level of resource availability the intensity of competition may moderate as defence of the resource becomes uneconomical (Grant et al., 2000). Experimental studies have confirmed the role of high male density (De Boer, 1981), low resource availability (Almada et al., 1995), and a combination of high male density and low resource availability on increasing male aggressive behaviour (Kanoh, 2000). On the other hand, to avoid a lower reproductive success males may have to face a trade off between aggressive defence of a territory and behaviours necessary to attract females. In *R. ocellatus* the intensity of courtship influences female mate choice (Casalini et al., 2009) and courtship rate has been demonstrated to increase when male density is low (De Boer, 1981; Kanoh, 2000; Spence & Smith, 2005).

In addition, density itself, defined as the number of males present per unit area, may not be the most appropriate measure of competition for resources. Lloyd (1967) proposed a measure termed 'mean crowding'; the number of conspecifics an individual encounters in competition for a resource. The mean crowding of competitors is a more meaningful measure than density or resource availability, since it encapsulates a measure of absolute density, the degree of clustering of individuals, and in the context of competition over fertilisations, the degree of skew among reproducing males and females (Pomfret & Knell, 2008).

Breeding resource availability has a major influence on sex roles and other features of animal mating systems (Emlen & Oring, 1977). For example, when nest sites are scarce it is often females and not males that initiate courtship (Almada et al., 1995; Borg et al., 2002). The evolution of polyandry in particular is associated with limited breeding resources for females, including the availability of paternal care (Maynard Smith, 1977; Clutton-Brock, 1989; Ligon, 1999). Intersexual competition is also predicted to occur predominantly within the sex that is 'qualified to mate' (i.e., the sex that has achieved sexual maturity and has the resources necessary for mating) and whose reproductive success is restricted by a lower number of qualified mates (Ahnesjö et al., 2001).

Here mean crowding, defined as the number of males per unit of resource, and density, defined as the number of individuals present per unit area, were independently manipulated to investigate their effect on male reproductive competition using the rose bitterling (*Rhodeus ocellatus*), a fish with a promiscuous, resource-based mating system. To do so males were exposed to combinations of high and low levels of crowding and density, while the operational sex ratio (OSR), the ratio of males and females ready to mate and an important determinant of mating competition (Jirotkul, 1999), was held constant. It was predicted that (1) male aggression would increase at high density and crowding, while courtship rate would decrease and (2) the frequency of alternative mating tactics would increase at high density and crowding. Crowding and density were expected to influence the proportion of mussels defended since at high crowding and low density territorial males are more likely to be able to monopolise a higher proportion of resources. Thus, since a change in the availability of resources and the number of rivals might affect male behaviour, a final prediction was that (3) there would be an interaction in the effects of crowding and density.

Bitterling are small freshwater fishes from East Asia (Cyprinidae, Acheilognathinae), with a single species-complex also present in Europe (Van Damme et al., 2007; Zaki et al., 2008). During the spawning season female bitterling develop a long ovipositor that they use to insert their eggs into the gill cavity of live unionid mussels through the mussel exhalant siphon, while the eggs are fertilized by the male releasing sperm over the inhalant siphon of the mussel (Duyvené de Wit, 1955). Dominant males aggressively defend territories to monopolize mussels, and court females to attract and lead them to a mussel for spawning (Smith et al., 2004). Male dominance is determined by size (Casalini et al., 2009), with smaller males adopting alternative mating strategies, which are conditional (Candolin & Reynolds, 2001; Smith et al., 2002). Territorial males may also steal fertilisations in the territories of neighbouring males (Smith et al., 2004). Bitterling are ideal candidates for examining the effects of crowding and density on male mating behaviour and reproductive success as their spawning site can be readily quantified and manipulated and they readily adapt to laboratory conditions. For a full review of bitterling reproductive ecology, see Smith et al. (2004).

Methods

Fish for experimental work were first generation offspring of, 200 wild caught *R. ocellatus* collected from the River Yangtze Basin, China, in 2005. Experimental fish were raised in captivity and were 18–24 months old when experiments were conducted. Prior to experiments fish were kept in stock aquaria measuring 60 (length) × 40 (width) × 40 (depth) cm. Stock aquaria were on a recirculating system with water temperature at 19°C. Each stock aquarium contained a 2 cm layer of sand substrate, a freshwater mussel and artificial plants as refuges. The fish were kept under a 16 h : 8 h light : dark regime and fed commercial flake fish food twice each day and a mixture of frozen chironomid larvae and live zooplankton 2–3 times each week. Freshwater mussels used in trials were *Unio pictorum*. This mussel occurs across Eurasia and is readily used as a spawning site by *R. ocellatus* (Casalini, 2007). Mussels were collected from the River Cam in Cambridgeshire, UK, stored in 160-l tanks and fed live phytoplankton daily.

Prior to experiments 96 males were haphazardly selected from stock aquaria, assigned by eye to three broad size categories, and individually marked with coloured elastomer tags (Northwest Marine Technology). Tag colours were white, blue and green; red was avoided since this is the hue of male nuptial colouration. Size-sorted males were held in separate stock aquaria and allowed to settle for two days.

Crowding was controlled by varying the number of mussels available for spawning, while density was controlled by varying the number of males. At a high level of crowding the ratio of males to mussels was 2:1 and at a low level 1:2. Experiments were conducted in a large aquarium measuring 180 (length) × 148 (width) × 100 (depth) cm with a volume of 2400 l. Male density was varied between 2 (low density) and 6 fish (high density). To ensure that the OSR remained unchanged across all treatments the number of females ready to mate was either 1 (low density) or 3 (high density), generating a ratio of males to females ready to mate of 2:1 (Table 1). Mussels were arranged in a grid (4 or 12 mussels) or row (3 mussels) equidistant from each other; irrespective of the number of mussels present, the same spacing between adjacent mussels was maintained in each trial. When a single mussel was used it was placed at the centre of the experimental aquarium. Thus, there were four treatment groups: low fish density and low mussel density, high fish density and low mussel density, low fish density and high mussel density, high fish density and high mussel density.

The day before the beginning of each trial males to be tested and females in spawning condition (with a fully extended ovipositor) were haphazardly selected from stock aquaria and placed in the experimental aquarium. Experimental mussels, each in a sand-filled pot covered with a perforated plastic cup to allow inspection of the mussel but not spawning, were placed in the experimental aquarium. Artificial plants were provided as refuges. To replicate the natural size structuring seen in bitterling populations (Smith et al.,

Table 1. Experimental design: number of males, females and mussels at different levels of mean crowding and density.

Crowding treatment	Density treatment	Mussels	♂♂	♀♀	Mussels per ♂	Male density (m^{-2})	OSR
Low	Low	4	2	1	2.0	0.75	2:1
High	Low	1	2	1	0.5	0.75	2:1
Low	High	12	6	3	2.0	2.25	2:1
High	High	3	6	3	0.5	2.25	2:1

Operational sex ratio (OSR) was male male-biased (2:1) in all treatments.

2000b), males in low-density trials (2 fish) were used from the large and small size classes, and in the high density trials 2 males from each size class (large, medium and small) were used. Body length (mean ± SE) in different size categories was: large 54.3 ± 0.36 mm, medium 47.0 ± 0.31 mm and small 41.3 ± 0.33 mm.

On the morning following stocking the mussels were uncovered and, after 1 h, the behaviour of every male was scored for 20 min using a palm computer with the FIT-system behaviour recording software (Held & Manser, 2005). Mussels were again covered for two hours and the procedure repeated in the afternoon, with male behaviour recorded for 20 min. After completion of a trial all mussels and fish were removed and a new group stocked. Thus, one replicate, with all combinations of crowding and density, was completed over a 4-day period. Treatment order and the order of focal male observations were randomized prior to the beginning of the experiment.

The behaviours scored were: (i) frequency of focal male aggressive defence of a mussel, (ii) frequency of aggression directed at the focal male, (iii) frequency of focal male courtship and (iv) frequency of focal male ejaculation. In addition, a record was made of the mussels defended by focal males and the mussels over which they ejaculated. These data enabled estimation of the proportion of available mussels that were defended, and the proportion into which males ejaculated, an index of the distribution of ejaculates and measure of the frequency of alternative male mating tactics.

After completion of a trial, the body length (from the tip of the snout to the base of the tail fin) of every male was measured to the nearest 1 mm and fish were returned to a stock aquarium and were not used again. Experimental mussels were measured to the nearest 1 mm (maximum shell length) and were not used again. A total of 6 independent replicates, each comprising all combinations of crowding and density, were completed using 96 males, 48 females and 120 mussels. Mean male body length did not vary significantly among treatment groups (one-way ANOVA: $F_{3,20} = 1.32$, $p = 0.294$).

Ethical note

Elastomer tags have been widely used to mark fish and have proven to be harmless (Halls & Azim, 1998; Malone et al., 1999; Imbert et al., 2007;

Weston & Johnson, 2008), which also proved the case in the present study. Aggressive territoriality is a normal feature of male bitterling spawning behaviour, though fish do not inflict physical damage on one another. The high-density experimental treatment in the current experiment consisted of only 6 males in a 2400-l aquarium, within the range of densities seen under natural conditions (Smith et al., 2000a). The test aquarium was furnished with artificial plants as refuges where attacked fish could readily avoid confrontation. Fish were constantly monitored before and during the experiment and none (or any in 15 years of experimental research) showed marks or injuries, or any signs of stress, during territorial disputes.

Data analysis

All data were tested for normality using a Kolmogorov–Smirnov 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. Each datum for individual male behaviour referred to the mean of each morning and afternoon, 20-min observation period. A one-way ANOVA was used to test for differences in male size among replicates. A Spearman's correlation was used to test the correlation between aggression rate and the mean proportion of mussels defended, between male size and net aggression rate, and between ejaculation rate and the mean proportion of mussels into which sperm was released. A two-way ANOVA was used to test the effect of crowding and fish density on mean male aggression rate, mean male ejaculation rate, mean sneaking rate, mean courtship rate, mean proportion of mussels defended, and mean proportion of mussels into which males ejaculated.

Results

There was a highly significant correlation between each male net aggression rate (number of aggressions performed minus mean number of aggressions received) and their size (Spearman's correlation: $r_{94} = 0.732$, $p < 0.001$; Figure 1). Bigger males performed more aggression and smaller males were attacked more frequently.

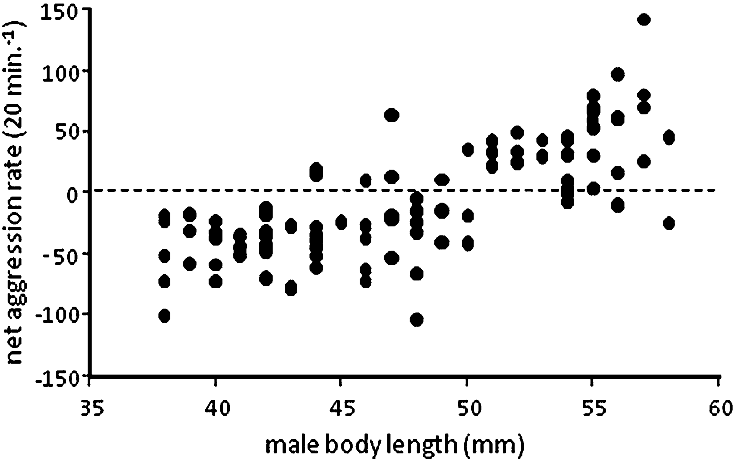

Figure 1. Net aggression rate (per 20 min), estimated as mean number of aggressions performed minus mean number of aggressions incurred as a function of male body length (mm).

Mean aggression rate and the mean proportion of mussels defended by males were not correlated (Spearman's correlation, $r_{22} = -0.020$, $p = 0.925$). There was no significant effect of either crowding or density or an interaction between variables on mean male aggression rate (rank transformed data, two-way ANOVA, crowding: $F_{1,20} = 3.75$, $p = 0.067$; density: $F_{1,20} = 1.15$, $p = 0.296$; interaction: $F_{1,20} = 0.21$, $p = 0.651$; Figure 2A), on mean male ejaculation rate (two-way ANOVA, crowding: $F_{1,20} = 0.05$, $p = 0.833$; density: $F_{1,20} = 0.17$, $p = 0.681$; interaction: $F_{1,20} = 2.81$, $p = 0.109$), and on mean male sneaking rate (square root transformed data, two-way ANOVA, crowding: $F_{1,20} = 0.06$, $p = 0.801$; density: $F_{1,20} = 2.03$, $p = 0.170$; interaction: $F_{1,20} = 0.18$, $p = 0.674$). However, there was a significant effect of both variables on the mean proportion of mussels defended by males (two-way ANOVA, crowding: $F_{1,20} = 11.83$, $p = 0.003$; density: $F_{1,20} = 14.89$, $p = 0.001$); a greater proportion of available mussels were defended at high levels of crowding and at low male density. There was no significant interaction between the variables ($F_{1,20} = 0.57$, $p = 0.459$; Figure 2B).

The proportion of mussels into which males ejaculated was used as an index of the distribution of ejaculates among mussels and, therefore, a measure of the incidence of alternative male mating tactics. Crowding but not density significantly affected the mean proportion of mussels into which males released sperm (two-way ANOVA, crowding: $F_{1,20} = 5.55$, $p = 0.029$;

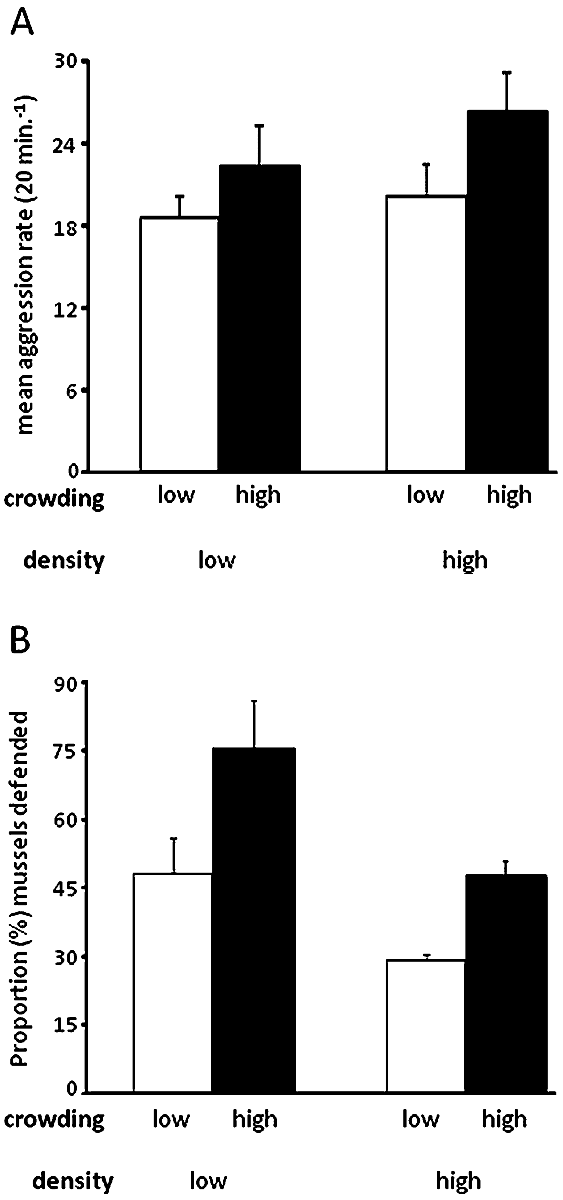

Figure 2. (A) Mean + SE aggression rate (per 20 min) and (B) mean + SE proportion (%) of mussels defended at low and high density and crowding.

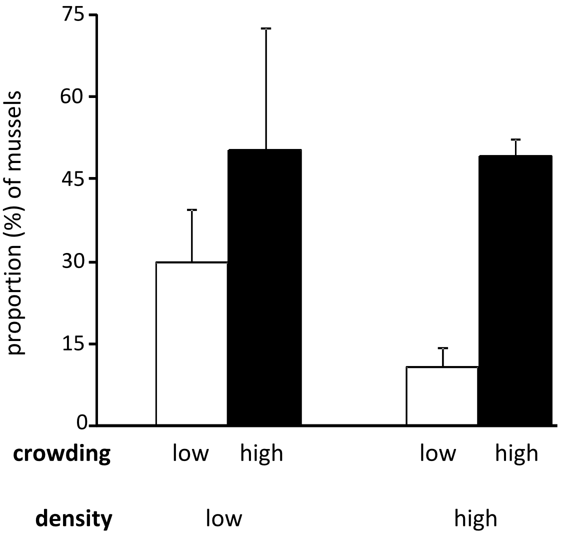

Figure 3. Mean + SE proportion (%) of mussels in which sperm was released at low and high density and crowding.

density: $F_{1,20} = 0.66$, $p = 0.425$; Figure 3), with the proportion of mussels higher at high crowding irrespective of density. There was no significant interaction between variables ($F_{1,20} = 0.54$, $p = 0.470$). The mean rate of ejaculation and the proportion of mussels into which sperm was released were significantly correlated (Spearman's correlation, $r_{22} = 0.619$, $p = 0.001$).

In contrast, density but not crowding significantly influenced mean male courtship rate. Mean courtship rate was lower at high density (two-way ANOVA: $F_{1,20} = 5.79$, $p = 0.026$; Figure 4). However, while mean courtship rate was also numerically lower with high crowding, this effect was not significant, though it approached significance ($F_{1,20} = 3.97$, $p = 0.060$). There was no interaction between variables ($F_{1,20} = 0.15$, $p = 0.705$).

Discussion

The aim of this study was to investigate the effects of two measures of resource competition on components of sexual selection in the rose bitterling, *R. ocellatus*, a fish with a resource-based mating system. The measures of resource competition used were male crowding (*sensu* Lloyd, 1967) and

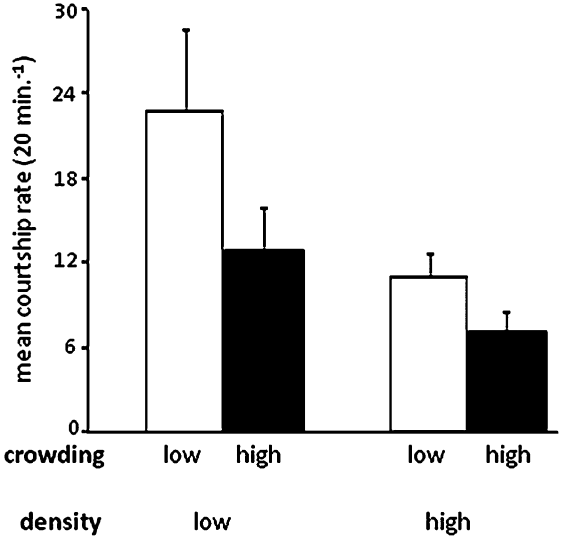

Figure 4. Mean+SE male courtship rate (per 20 min) at low and high density and crowding.

density. It was predicted that male aggression would increase at high density and crowding, while courtship rate would decrease, and that the frequency of alternative mating tactics would increase at high density and crowding. A final prediction was that crowding and density would interact.

Surprisingly, neither crowding nor density affected overall levels of male aggression. Aggression among males was shown to be dictated strongly by body size; bigger males tended to perform more aggression and smaller males were attacked more frequently. Body size in the related *R. amarus* also determined male lifetime reproductive success (Reichard et al., 2009). This size effect appears to arise through male control of spawning resources and success in sperm competition (Reichard et al., 2009). An alternative means of understanding the pattern of male-male interactions with respect to density and crowding would be to utilise social network analysis (Croft et al., 2008). This approach would serve to identify whether aggressive interaction among males were distributed randomly among a network of interacting males, or where concentrated on specific individuals, for example among a subset of larger males.

Density, but not crowding, had a significant effect on male courtship rate. At high fish density, courtship rates were lower, possibly the result of a trade-off between defending resources from rivals and attracting mates. This trade-off has been found in previous studies in bitterling (Reichard et al., 2004b), as well as in guppies (*Poecilia reticulata*) (Jirotkul, 1999), three-spined sticklebacks (*Gasterosteus aculeatus*) (Le Comber et al., 2001), and zebrafish (*Danio rerio*) (Spence & Smith, 2005). This result suggests that while male courtship rate is sensitive to a change in the number of rivals, access to resources has more trivial significance, though note that the pattern of effect of crowding on courtship rate mirrored that of density and the result approached significance. In addition to the rate of courtship, the intensity of courtship may by modulated by males and has been demonstrated to play an important role in influencing female mate choice, and consequently the strength of sexual selection in several taxa, including bitterling (Reichard et al., 2005; Casalini et al., 2009), the pacific blue-eye (*Pseudomugil signifier*) (Wong, 2004), the guppy (Matthews et al., 1997), the bicolor damselfish (*Stegastes partitas*) (Knapp & Kovach, 1991) and the katydid (*Requena verticalis*) (Gwynne, 1984). Courtship intensity was not measured in the present study, though it might be sensitive to changes in density or crowding. Female willingness to spawn or response to male courtship may have influenced male behaviour but, in the present experiment, the number of spawnings was rare irrespective of mussel crowding or fish density. Also, as females were not marked, it was not possible to test the number of spawning events for each individual female when more than one female was present.

Crowding significantly affected the proportion of mussels defended by males, a measure of male response to rival males adopting alternative mating tactics, with more mussels defended at a high level of crowding. Crowding also affected the success of alternative male mating tactics, measured as the proportion of mussels into which sperm was released by males, with males ejaculating into a higher proportion of mussels at a high level of crowding. Thus, for both these variables an elevated level of crowding had the effect of increasing the intensity of competition among males, either directly for spawning sites or through risk of sperm competition. While the effect of crowding appears predictable with hindsight, this study demonstrates first that, in rose bitterling at least, when resources essential for reproduction were available to males they are used. Second, that crowding and density are two distinct variables, though in the literature density is commonly used as a proxy for crowding.

Male control of spawning sites and distribution of ejaculates are significant determinants of male reproductive success in bitterling (Smith et al., 2003; Reichard et al., 2004a, 2005; Casalini et al., 2009) and other species (Alonzo & Warner, 2000; Wedell et al., 2002; Spence & Smith, 2005; Taborsky, 2008). The significant effect of crowding on these variables suggests that crowding around resources critical for reproduction may influence the strength of intrasexual selection. A consequence of this result is that where male crowding is high, intrasexual selection will be strong and traits that benefit males in competition for resources will tend to increase in frequency. In the case of bitterling these traits include large body size, red colouration in the eye and on the fins, and aggressive behaviour (Smith et al., 2003; Reichard et al., 2005; Casalini et al., 2009). Conversely, where crowding around resources is low the prediction is for relatively small and drab males that do not engage in aggressive contests. Under these conditions the mating system would be one in which females would be able to exercise mate choice with little constraint from male dominance and resource monopolisation. Bitterling offer an unusually tractable model for investigating these predictions. Previous work has shown that bitterling occur in lakes in which their population size can vary 90-fold and mussel abundance 500-fold (Smith et al., 2000a). Consequently, crowding may vary over several orders of magnitude under natural conditions among bitterling populations, with predictable impacts on mating system evolution among populations.

The key finding of the present study was that male behaviour appeared more sensitive to crowding; the number of conspecifics an individual encounters in competition for a resource, than to density per se. Density, particularly measured simply as the number of individuals per unit area, may fail to capture the degree of competition for resources, with consequent unpredictable responses to density (Kokko & Rankin, 2006). Future research should explore the effect of intrasexual selection on male adaptive responses to variation in crowding in natural populations, with an approach utilising social network analysis one that may be especially rewarding.

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