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Adjustments of ejaculation rates in response to risk of sperm competition in a fish, the bitterling (*Rhodeus sericeus*)

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Game theory models of sperm competition predict that within species, males should increase their sperm expenditure when they have one competitor, but decrease expenditure with increasing numbers of competitors. So far, there have been few tests or support for this prediction. Here, we show that males of a freshwater fish, the European bitterling, *Rhodeus sericeus*, do indeed adjust their ejaculation rate to the number of male competitors by first increasing and then decreasing their ejaculation rates as the number of competitors increases. However, this occurred only under restricted conditions. Specifically, the prediction was upheld as long as no female had deposited eggs in the live mussels that are used as spawning sites. After one or more females had spawned, males did not decrease their ejaculation rates with the number of competitors, but instead they became more aggressive. This indicates that decreased ejaculation rate and increased aggression are alternative responses to increased risk of sperm competition.

Keywords: alternative reproductive behaviours; mate guarding; sexual selection; trade-offs; Cyprinidae; Unionidae

1. INTRODUCTION

Game theory models predict that sperm expenditure should depend on the intensity of sperm competition (Parker *et al.* 1996; Ball & Parker 1997; Parker 1998). The relationship should, however, differ among and within species. While sperm expenditure should increase with the intensity of sperm competition across species, theory predicts that within species a male's expenditure should increase when there is one competitor, but then decrease as additional males compete. This predicted reduction within species stems from decreasing marginal gains per unit of expenditure as competition increases (Parker *et al.* 1996).

The prediction of increased sperm expenditure with sperm competition across species is well supported (Møller 1991; Gage 1994; Parker *et al.* 1997; Stockley *et al.* 1997; Birkhead & Møller 1998), but different patterns have been found within species. While some cricket species adjust their sperm expenditure to the intensity of sperm competition in accordance with the theory, others do not (Simmons & Kvarnemo 1997; Schaus & Sakaluk 2001). Moreover, in a fish species, the rainbow darter, *Etheostoma caeruleum*, males do not tailor their ejaculates to the intensity of sperm competition, but instead forego spawning more often under high compared with low sperm competition intensity (Fuller 1998). In the white butterfly, *Pieris rapae*, only mated males adjust their ejaculate size to the intensity of sperm competition whereas virgin males do not (Wedell & Cook 1999).

An alternative to adjusting sperm expenditure to the number of competing males is to invest in the prevention of sperm competition (Alonzo & Warner 2000). Some support for this possibility has been found in a few fish species where dominant males increase their investment into mate guarding at the cost of gamete production when the risk of sperm competition is high (Warner *et al.* 1995; Marconato & Shapiro 1996; Warner 1997; Alonzo & Warner 2000).

Here, we examined relationships between male ejaculation rates and numbers of competitors in a freshwater fish species, the European bitterling, *Rhodeus sericeus*, (Cyprinidae). We relate the findings to the level of aggression to determine how adjustment of sperm expenditure relates to investment into the prevention of sperm competition. In bitterling, some males establish territories around one or more living freshwater mussels (Unionidae), while others, which are usually smaller and younger, participate in mating by sneaking. Females lay two to four eggs at a time into the gills of a mussel by rapidly inserting a long ovipositor into the exhalent siphon. Males fertilize the eggs by releasing sperm over the inhalent siphon both before and after egg laying (Wiepkema 1961; Mills & Reynolds 2002*a*). Bitterling sperm are short-lived (a maximum of 4 min (Kanoh 1996)), as is typical for freshwater fishes (Billard 1986), and males typically release sperm one to four times per minute during courtship (Candolin & Reynolds 2001). Females often spend several minutes inspecting a mussel before spawning, and are highly selective (Smith *et al.* 2000; Mills & Reynolds 2002*a*,*b*). We have shown elsewhere that males become more aggressive after a female has spawned in a mussel (Candolin & Reynolds 2002).

We determined the effects of numbers of competitors on ejaculation rates by dominant and subdominant males during two sequential female spawnings when dominant males differed in their aggression level. The study formed part of an investigation of the effect of male density on male and female reproductive behaviour (Candolin & Reynolds 2002). Because ejaculation rate does not give

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the absolute number of sperm released, we tested whether it was correlated with sperm expenditure by measuring the size and density of the sperm clouds seen during each ejaculation.

2. METHODS

Bitterling and mussels, *Anodonta anatina*, were collected from Reach Lode, a slow-flowing canal in Cambridgeshire, UK (see Reynolds *et al.* 1997; Reynolds & Guillaume 1998). They were kept separately in large aquaria (120 cm × 60 cm, 40 cm high) in the laboratory. The temperature of the water was increased gradually from 12 to 18 °C and the light cycle matched the natural cycle.

The experiment was carried out in four aquaria (120 cm × 60 cm × 40 cm) containing one mussel and either one, two, four or six males which ranged in size from 50 to 61 mm standard length and which had been chosen randomly for each replicate (see Candolin & Reynolds (2002) for details). One day after males had been introduced into the aquaria, a single female in spawning condition was introduced sequentially into each of the four male density treatments. We alternated the order among replicates. Thus, the experiment conformed to a randomized block design with the four different male densities as treatments and females as the blocking factor. The female was given a 2 h resting period after she had been in each experimental aquarium. In each case the female was first enclosed in a net bag to acclimatize her to the aquarium, and then released after 1 min, whereupon the fishes were video-filmed. Trials lasted until 2 min after spawning or until 15 min had elapsed since the female first inspected the mussel in cases where the female did not spawn within this time period (two cases). Males were courting the female and releasing sperm in the two cases where the female did not spawn.

A second female was introduced 1 h after the first female was removed from each aquarium and the same procedures were followed, i.e. the female was transferred among all four male density treatments, with a 2 h rest interval. Thus, each female was tested four times (four male densities) and each set of males was tested twice (first and second female). We had 15 replicates with different individual fish and mussels.

The ejaculation rates by the dominant and subdominant males before oviposition and 2 min after oviposition were recorded from the tapes. In aquaria with several males, we defined the dominant, territorial male as the one that chased away the other males after the female had spawned. He was usually the largest male and had the reddest nuptial coloration (Candolin & Reynolds 2001). Solitary males always adopted the territorial, courting tactic.

To estimate whether ejaculation rate reflects sperm expenditure, the size and density of the sperm clouds seen immediately after each ejaculation was determined. The seminal fluids of fishes are transparent and the whiteness of the ejaculate therefore depends on sperm number (M. B. Rasotto, personal communication; Mazzoldi *et al.* 2000; Rasotto & Mazzoldi 2002). To measure the sperm clouds, the film was digitized and a box was drawn around each sperm cloud. The box was large enough to encompass the largest sperm cloud observed. The same sized box was drawn around each sperm cloud. We then measured the mean grey value of the selected, constant area by using ImageJ v. 1.26 (http://rsb.info.nih.gov/ij). The cloud was seen against a dark background, and the light conditions and the background were the same in each aquarium. This method accounts for differences in the volume of ejaculates and therefore approximates the total number of sperm released. Some ejaculates could not be measured, due to another fish blocking the view or forming part of the background, but the number of ejaculates that had to be excluded never exceeded one-third of all ejaculates of a fish. This method is not as accurate as direct sperm counts, but these could not be done without disrupting the fish.

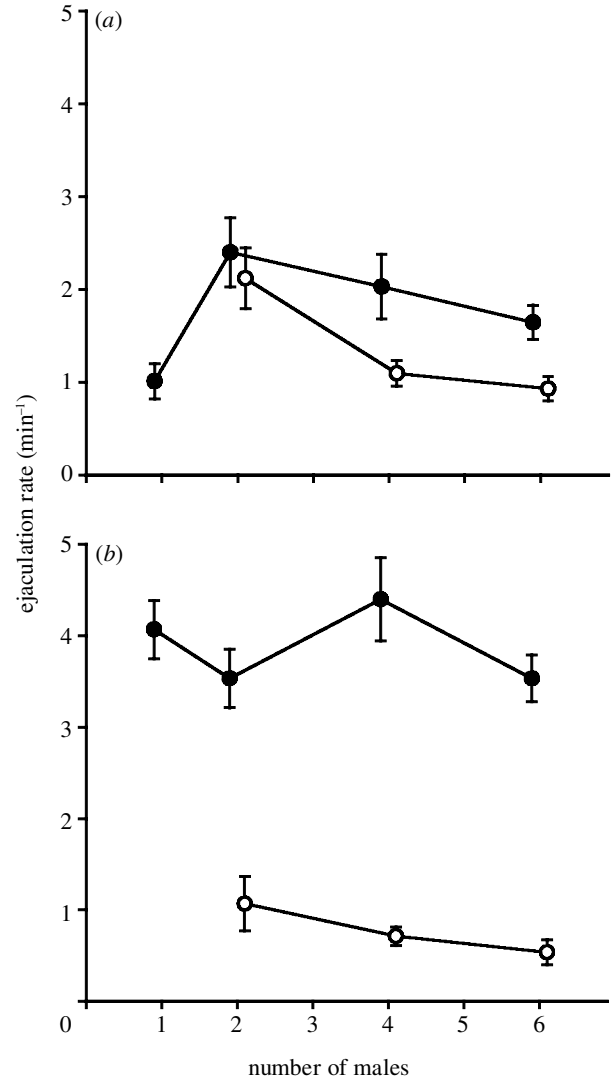

Figure 1. Ejaculation rate (mean ± s.e) of single/dominant males (filled circles) and per individual of other males (open circles) during the first female presentation: (*a*) before oviposition and (*b*) after oviposition.

3. RESULTS

As predicted by the theory, the ejaculation rate of the dominant male showed a curvilinear relationship to male density during the first female presentation: the rate of ejaculation was low when no other males were present, higher when one other male was present, and was progressively lower at densities of four and six males (figure 1*a*). This curvilinear relationship with density is demonstrated by a significant quadratic contrast term in a polynomial model of the effect of male density on ejaculation rate (mixed model ANOVA with male density as fixed factor and female as random factor: $F_{3,42} = 4.49$, $p = 0.008$; quadratic contrast term $= -0.88$, s.e. $= 0.28$, $p = 0.003$).

Male density influenced ejaculation rate of the dominant male, as shown by the significant *F*-value. The linear and cubic contrast terms were non-significant ($p > 0.1$). The total ejaculation rate by the other, subdominant, males increased linearly with the density of males ($F_{2,28} = 7.08$, $p = 0.003$; linear contrast term = 1.78, s.e. = 0.48, $p < 0.001$), but ejaculation rate per subdominant male decreased with density (figure 1*a*; $F_{2,28} = 8.17$, $p = 0.002$; linear contrast term = −0.84, s.e. = 0.23, $p = 0.001$).

After the female had spawned, the ejaculation rate of the dominant male no longer depended on male density (figure 1*b*; $F_{3,42} = 1.58$, $p = 0.21$). This was a significant change from the pattern before oviposition (interaction term: $F_{3,42} = 4.14$, $p = 0.012$). The ejaculation rate per subdominant male also no longer depended on male density ($F_{2,28} = 2.07$, $p = 0.14$), although the pattern did not differ statistically from the one before oviposition (interaction term: $F_{2,28} = 2.02$, $p = 0.15$). The ejaculation rate of the subdominant males had decreased compared with before oviposition ($F_{1,14} = 10.24$, $p = 0.006$).

During the second female presentation, the dominant male's ejaculation rate before oviposition no longer depended on the density of males (figure 2*a*; $F_{3,42} = 1.27$, $p = 0.30$), but the pattern did not differ statistically from the first spawning before oviposition (interaction term: $F_{3,42} = 0.84$, $p = 0.48$). The ejaculation rate of the subdominant males decreased compared with the first spawning ($F_{1,14} = 15.74$, $p < 0.001$), but the ejaculation rate per subdominant male still decreased with male density ($F_{2,28} = 5.23$, $p = 0.012$). After oviposition, the ejaculation rate of the dominant male did not depend on male density ($F_{3,40} = 1.05$, $p = 0.38$), but ejaculation rate per subdominant male decreased with male density ($F_{2,26} = 8.87$, $p < 0.001$; the decrease in degrees of freedom is due to two cases where the female did not oviposit).

The density of the sperm clouds of dominant males did not differ among male densities during the first spawning, either before oviposition (mean (± s.e.) grey value of pixels when one male: 86.3 ± 2.7, two males: 78.6 ± 2.4, four males: 80.3 ± 3.2, six males: 76.4 ± 3.1; $F_{3,43} = 1.24$, $p = 0.31$) or after oviposition (one male: 74.0 ± 2.9, two males: 66.0 ± 2.7, four males: 70.6 ± 2.9, six males: 70.1 ± 2.7; $F_{3,49} = 1.48$, $p = 0.23$). The same lack of relationship held during the second spawning before oviposition (one male: 81.9 ± 2.8, two males: 73.5 ± 2.4, four males: 77.2 ± 3.2, six males: 78.7 ± 3.5; $F_{3,45} = 1.84$, $p = 0.15$) and after oviposition (one male: 79.0 ± 3.5, two males: 68.9 ± 2.6, four males: 79.0 ± 4.1, six males: 74.0 ± 4.7; $F_{3,44} = 1.69$, $p = 0.18$).

A negative relationship occurred between changes in aggression from the first to the second spawning before oviposition and changes in ejaculation rate of the dominant male; the more aggressive the male became, the larger was his reduction in ejaculation rate, although only a trend towards a negative relationship was found at the highest density, as ejaculation rate had been low already during the first female presentation (figure 3; two males: $r^2 = 0.60$, $F_{1,14} = 19.5$, $p = 0.001$; four males: $r^2 = 0.45$, $F_{1,14} = 10.5$, $p = 0.006$; six males: $r^2 = 0.24$, $F_{1,14} = 4.20$, $p = 0.061$). No significant relationships were found between changes in aggression and changes in ejaculation rate from before to after oviposition either during the first or the second spawning (all p values > 0.10). Males became highly aggressive for a few minutes immediately after the female had spawned (see Candolin & Reynolds 2002) and yet maintained a high ejaculation rate, which was not adjusted to the number of competitors.

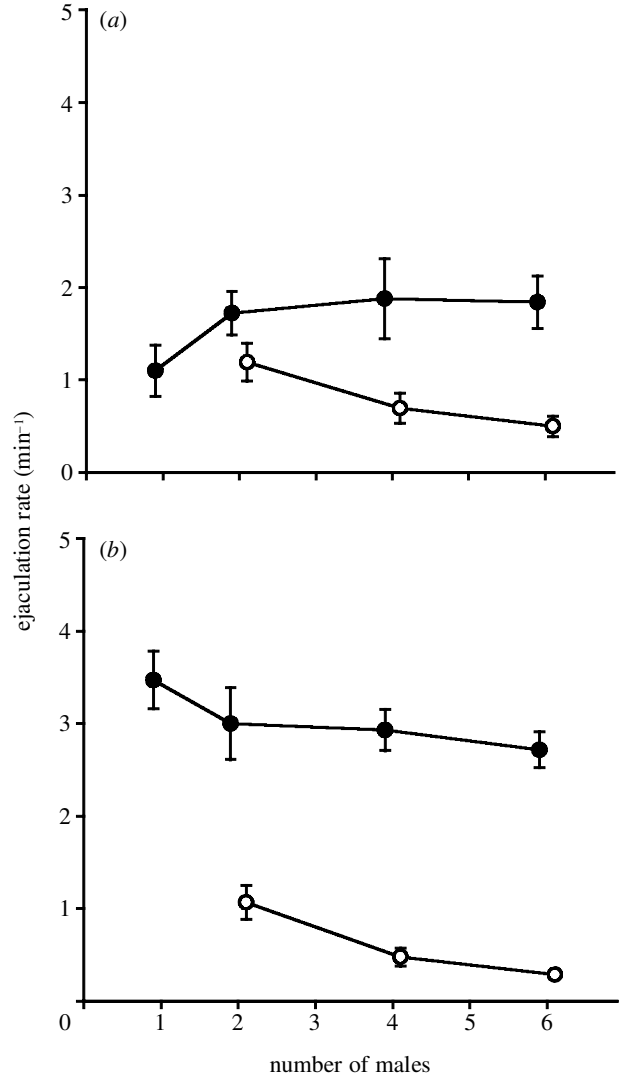

Figure 2. Ejaculation rate (mean ± s.e.) of single/dominant males (filled circles) and per individual of other males (open circles) during the second female presentation: (*a*) before oviposition and (*b*) after oviposition.

4. DISCUSSION

Male bitterling adjusted their ejaculation rates to the density of competing males before the first female spawned, in support of the game theory model of sperm competition proposed by Parker *et al.* (1996). As predicted, the rate of ejaculation by the dominant male reached a peak when only one other male was present and then decreased with the number of competitors. The reduction in ejaculation rate when more than one competitor was present may have been due to a decreased benefit per ejaculate as the number of males increased beyond two, as suggested by Parker *et al.* (1996). Although we did not measure sperm expenditure directly, we did not find any difference in the size and density of the sperm clouds released by the dominant male when in the presence of different numbers of competitors.

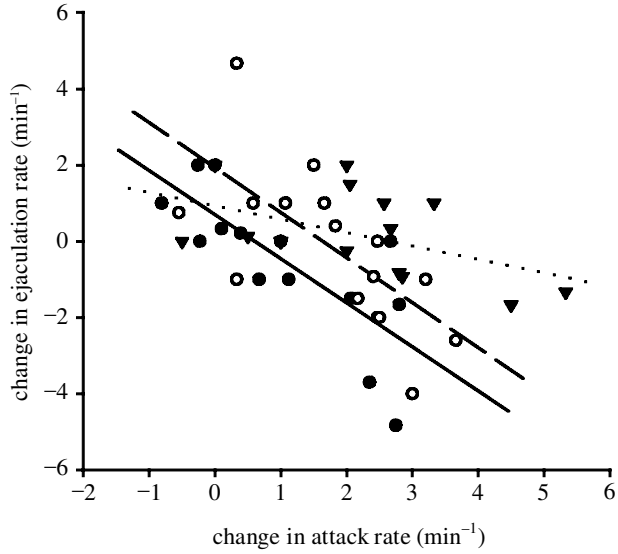

Figure 3. Relationship between changes from the first to the second female presentation in attack rate and ejaculation rate of the dominant male at different male densities: two males (filled circles, solid line: $y = 0.64 - 1.15x$), four males (open circles, broken line: $y = -1.88 - 1.16x$), and six males (filled triangles, dotted line: $y = 0.95 - 0.35x$).

Together with findings for some cricket species and for white butterfly males (Simmons & Kvarnemo 1997; Wedell & Cook 1999; Schaus & Sakaluk 2001), our results show that under some circumstances males adjust their ejaculation behaviour in the manner predicted by sperm competition theory, although the examples are few.

The patterns of sperm release of the dominant male did not follow the theory's predictions after the first female had spawned, nor before or after a second female spawned. Our companion paper has shown that dominant males become highly aggressive to other males after oviposition and remain aggressive during a second spawning (Candolin & Reynolds 2002). The data reported here show that the ejaculation rate of the other males then decreases. These results indicate that increased aggression and adjusted ejaculation rate are two alternative responses by territorial males to increased risk of intense sperm competition. This interpretation is supported by increases in aggression being matched by decreases in ejaculation rates by the dominant male from the first to the second female presentation. A trade-off between aggression (mate guarding) and sperm expenditure has been found in a few other fish species (Warner *et al.* 1995; Marconato & Shapiro 1996; Warner 1997; Alonzo & Warner 2000), which suggests that aggression and sperm production are expensive in terms of time or energy. After oviposition, dominant males appear to be able to maintain both a high ejaculation rate and a high aggression level for a few minutes when the eggs can still be fertilized. Male aggression before oviposition did not increase with male density above two (Candolin & Reynolds 2002) and is therefore not the proximate explanation of the decrease in the dominant males' ejaculation rate when more than two males were present.

Why do dominant males adjust their ejaculation rate to the presence of competing males before the first female spawns but invest in aggression afterwards? Alonzo & Warner (2000) predicted that males should guard mates when the risk of sperm competition is high if this behaviour prevents sperm competition and does not greatly reduce sperm production, assuming a trade-off between sperm production and guarding. This model did not consider the potential influence of a cost of aggression in mate attraction. This is important in bitterling, because aggression against other males interrupts courtship and increases the time until a female spawns, whereas the presence of other males around a mussel leads to more rapid attraction of females (Candolin & Reynolds 2002). Thus, during the first female presentation, low aggression by the male may have increased the probability that the female would spawn in the mussel, leaving adjustments in ejaculation frequency as the best way of combating sperm competition. After the female had spawned, there were no benefits to gain by tolerating other males and the male became highly aggressive. Dominant males still maintained a high ejaculation rate but did not adjust it to the presence of other males. Males may therefore have been exhibiting a form of postcopulatory mate guarding.

In conclusion, the results support the theory that males should respond to intense sperm competition by adjusting their ejaculation rate to the number of competing males. The next step is to examine paternity in relation to numbers of sperm released, to examine the effectiveness of this tactic.

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