PROCEEDINGS OF THE ROYAL SOCIETY B

Proc. R. Soc. B (2005) **272**, 1683–1688
doi:10.1098/rspb.2005.3140

Females solicit sneakers to improve fertilization success in the bitterling fish (*Rhodeus sericeus*)

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In most species there is greater variance in reproductive fitness among males than females. A consequence is the evolution of alternative male mating tactics, including sneaking behaviour whereby males attempt to mate with females courted by other males, which could undermine female choice. In bitterling (*Rhodeus sericeus*), fish that lay their eggs on the gills of living freshwater mussels, sneaking is common and successful. Here we show that female bitterling can significantly improve their fertility with an increase in the number of spawning partners. Females perform conspicuous behaviours associated with spawning more frequently close to sneakers, and spawn more eggs close to high-quality sneakers. This is the first time that females have been shown to engage in behaviour that increases the probability of sneaking, and raises the possibility of a sexual conflict over the number and source of ejaculates during spawning in this species.

Keywords: extra-pair copulations; sperm limitation; sperm competition; sneaking; strategic ejaculation

1. INTRODUCTION

Mating with multiple males can confer indirect benefits to females by increasing offspring fitness or direct benefits through insurance of fertilization (Jennions & Petrie 2000). Infertility rates in nature can be high (Eberhard 1996), and are often explained through sperm depletion in males (Wedell *et al.* 2002), following multiple ejaculations (Nakatsuru & Kramer 1982), or through genetic incompatibility (Birkhead & Møller 1998; Kempenaers *et al.* 1999; Tregenza & Wedell 2002). Males may tailor ejaculate size and frequency in response to the risk of sperm competition (Ball & Parker 1997), female mating status (Fuller 1998), female quality (Marconato *et al.* 1995), and prospect of further mating opportunities (Gómez & Serra 1996). If female fertility is limited by the availability of sperm, these conditions raise the possibility of an intersexual conflict over optimum ejaculate size and frequency, with the optimal tactic for males being one of prudent sperm allocation among females, while that for females is for more lavish sperm expenditure, by multiple males, that ensures fertilization (Warner *et al.* 1995; Alonzo & Warner 2000).

In some species females mate preferentially with males that demonstrate their superiority through possession of high quality resources and investment in energetically expensive courtship and sexual signalling (Andersson 1994). Other males engage in alternative mating tactics, thereby avoiding the costs associated with attracting mates. A common alternative-mating tactic is sneaking, when males attempt to fertilize eggs without investing in either attracting females or offspring care (Andersson 1994). Because sneaking inevitably erodes the reproductive success of males chosen by females (Avise *et al.* 2002; Reichard *et al.* 2004*a*), it also undermines female choice.

Here we investigate a female response to sneaking in the European bitterling (*Rhodeus sericeus*). Bitterling are fishes with a promiscuous, resource-based mating system. Males guard territories around living freshwater mussels and attract females to deposit their eggs in mussels in their territory. Females use long ovipositors to place their eggs onto the gills of a mussel through the mussel's exhalant siphon; males fertilize the eggs by releasing sperm into the inhalant siphon, and sperm must pass through the mussel gill to reach the eggs. Female bitterling base their mate choice on mussel quality and male size (Smith *et al.* 2001), with consistent preferences for certain mussel characteristics and for large males (Smith *et al.* 2000*a*,*b*; Reichard *et al.* 2005; reviewed by Smith *et al.* 2004). Most bitterling spawnings involve sneaking, and sperm expenditure per spawning is high (Smith *et al.* 2000*a*, 2001, 2002, 2003; Reichard *et al.* 2004*a*,*b*). Male mating behaviour is opportunistic, with no genetic or morphological distinction between territorial and sneaking males, though larger males are generally more successful in establishing territories than smaller males (Reichard *et al.* 2004*a*, 2005). Notably, even successful territory holders commonly invade adjacent territories to sneak (Smith *et al.* 2004). Consequently, sneaked fertilizations by male bitterling resemble aspects of extra-pair copulations (EPC) in birds, though bitterling do not form pairs or exhibit parental care. Males mate repeatedly each day throughout the breeding season (Smith *et al.* 2004), raising the possibility of sperm depletion, and it is notable that a high proportion of eggs may not be fertilized (Reichard *et al.* 2004*b*). Territorial males use the presence of a rival as a visual cue to detect the risk of sperm competition (Smith *et al.* 2003), directing aggression towards sneakers, strategically adjusting their ejaculation rate, and leading females to mussels where there is a lower risk of sperm competition (Smith *et al.* 2002, 2003; Reichard *et al.* 2004*a*,*b*).

Little is known about female spawning preferences and behaviour in the presence of sneakers. In this study we tested the prediction that fertilization success of female

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Received 28 December 2004
Accepted 25 April 2005

bitterling is higher if more males participate in a spawning. We also tested whether females were sensitive to the proximity of a sneaker during spawning and sneaker quality. We finally tested the prediction that females should signal an intention to spawn to increase the number of males that participate in a spawning, if mating with multiple males increases female fertility.

2. METHODS

(a) *Fertilization success*

We conducted a laboratory experiment to investigate whether fertilization success in *R. sericeus* varies as a function of the number of males that participate in a spawning. Male number was varied at two levels; high (four males) and low (single male). Fish for experimental work were collected by electrofishing in late April 2004 from the River Kyjovka in the southeast of the Czech Republic, where *R. sericeus* are abundant (Reichard *et al.* 2002). Experiments were performed in the aquarium facility at the Institute of Vertebrate Biology, Brno, Czech Republic during May 2004.

Either a single or group of four males were stocked in experimental aquaria measuring 75 (length) × 40 (width) × 40 (depth) cm. Fish were held under a natural light cycle and fed a mixture of live bloodworm (*Tubifex* spp.), live and frozen chironomid larvae, and commercial flake food. Water temperature in aquaria matched natural variations and varied between 18 and 21 °C. Each aquarium contained a 2 cm layer of sand substrate and two artificial plants as refuges. Experimental aquaria were isolated using opaque barriers so that fish in adjacent aquaria could not interact. One *Unio pictorum* mussel in a sand-filled flowerpot was placed in the centre of each aquarium. Mussels were collected in mid April (before the start of the bitterling spawning season) from an oxbow lake in which bitterling occurred, adjacent to the River Kyjovka, and transported to the Institute of Vertebrate Biology. They were stored in a shaded garden pond where phytoplankton food was abundant. The second day after stocking, a female bitterling with an extended ovipositor was introduced into the aquarium and observed until she spawned. After spawning, the fish were not disturbed for a further 8 min; in previous studies it was shown that ejaculations ceased after approximately 2 min (Smith *et al.* 2003). An interval of 8 min allowed sufficient time for ejaculations to cease and for eggs to be fertilized.

After 8 min the mussel was removed from the aquarium, opened and the recently deposited eggs carefully removed. The eggs were transferred to a plastic Petri dish containing aquaria water. After 6 h the eggs were scored for whether they had been fertilized. Fertilized eggs can readily be distinguished from unfertilized eggs because they develop a wide perivitelline space and undergo repeated cell divisions. After removal of the mussel, the males were captured, their standard length (from the tip of the snout to the origin of the caudal fin) measured, and were not used again in the experiment. The mean ± s.e. standard length of males in the high male treatment was 50 ± 0.9 mm and low 49 ± 1.6 mm, which was not significantly different. Females were transferred to a second aquarium assigned to the alternative male treatment. The exposure of females to male treatments was assigned according to a predetermined random order. Consequently, each female was exposed to both the high and low male treatments, giving a paired design for statistical analysis. After a second spawning the female was removed, measured and was not used again in the experiment. The mean ± s.e. standard length of females was 49 ± 1.1 mm. There was no significant difference in the length of mussels in the high and low male treatments; the mean ± s.e. length of mussels in the high male treatment was 73 ± 1.7 mm and low 75 ± 2.7 mm. During trials the total number of ejaculations by males was recorded, with a distinction drawn between pre-oviposition and post-oviposition ejaculations.

(b) *Female response to males*

This experiment investigated the effect of variation in the size of a territorial and rival male on the behavioural response and oviposition decisions of female bitterling. Experiments were conducted inside mesh observation cages placed in a large outdoor concrete pool at the Institute of Vertebrate Biology, Brno during May 2003. The pool measured 12.4 by 6.0 m and water depth was 0.6 m. Water temperature varied naturally between 17 and 24 °C. The pool was stocked with approximately 250 adult bitterling collected from the River Kyjovka 10 days before the start of the experiment. Fish in the pool were fed daily on frozen bloodworm, and a carpet of algae, the principal component of the natural diet of bitterling (Przybylski 1996), established on the walls and floor of the pool on which fish grazed.

Observation cages measured 150 (length) × 130 (width) × 70 (depth) cm and were constructed from rigid netting with a mesh size of 0.5 × 0.5 cm. Two sand-filled flowerpots and 2.5 l square-sided glass jar were placed inside the observation cage on opposite sides, such that each pair, comprising a flowerpot and jar, was 110 cm apart. A single *U. pictorum* was placed in each of the flowerpots. Two size classes of territorial and rival males were used; large (2 years old, 53 ± 0.7 mm), and small (1 year old, 39 ± 0.5 mm). Following a randomly predetermined order, a male bitterling of either size class was haphazardly captured with a hand net by a diver and released in the observation cage. After between 1 and 2 h the male had established a territory around both mussels and actively defended it.

To begin the experiment, a second male bitterling, its size class determined according to a randomly predetermined order, was confined in one of the glass jars to act as a rival. The position of the rival male, on either the left or right side of the cage, was randomly determined. A female with an extended ovipositor was haphazardly selected from the pool and added to the cage in a third 2.5 l glass jar placed in the centre. After 5 min the female was gently released from the jar. The behaviour of the territorial male and female was videoed through a gap in the wall of the cage by a diver until the female spawned. Once spawning occurred the behaviour of the territorial male was recorded for at least a further 2 min or until sperm releases ceased. After spawning, the mussels were removed, measured and the number of eggs deposited by the female recorded. The rival male in the glass jar and the female were removed and measured and were not used in further trials. The territorial male was then presented with a second male in a glass jar, another female with extended ovipositor, and another pair of mussels. Following a second spawning all the fish and mussels were removed and measured. The mean ± s.e. standard length of females was 47 ± 0.9 mm. There was no significant difference in the length of mussels adjacent to a bottled rival male, compared to mussels adjacent to an empty bottle; the mean ± s.e. length of mussels with a male adjacent was 80 ± 1.4 mm and without 81 ± 1.6 mm. Thus, each territorial male participated in two spawnings with a different female, confined rival male, and mussels. During experiments bottled male fish did not respond strongly to territorial males, though they did show occasional aggression to the territorial male and courtship behaviour to females. Bottled fish swam naturally during experiments, and did not show signs of distress during the time they were confined.

The behaviours scored from video recordings were: position of the mussels in which females spawned, frequency of sperm releases by the territorial male, rate of aggression directed towards the rival male by the territorial male, position of the mussel to which territorial males led females, rate of female mussel siphon inspection, rate of female skimming, and period after spawning that females remained within two body lengths of a mussel. For a description of behaviours see Smith *et al.* (2004). Behaviour was scored only for those trials in which a spawning took place.

3. RESULTS

(a) *Fertilization success*

The proportion of eggs fertilized was significantly different between treatments (paired *t*-test, $t_{14}=3.14$, $p=0.007$; figure 1*a*). The mean±s.e. difference in the proportion of eggs fertilized between treatments was 0.20±0.063. The total number of ejaculations was higher in spawnings with a group of males than with a single male (unpaired *t*-test, square-root transformation, $t_{28}=3.68$, $p=0.001$; figure 1*b*). There was a significant correlation between fertilization success and the frequency of pre-oviposition ejaculation (Pearson's correlation, $r_{1,28}=0.39$, $p=0.034$; figure 1*c*), but not post-oviposition ejaculation (Pearson's correlation, $r_{1,28}=0.28$, $p=0.137$; figure 1*c*). There was no significant difference in the number of eggs spawned by females between treatments (paired *t*-test, $t_{14}=1.82$, $p=0.089$). The mean±s.e. number of eggs spawned by females in the high male treatment was 4.7±0.30 and low 4.2±0.39.

(b) *Female response to males*

A total of 58 trials were conducted, of these spawning took place in 36. Females spawned more frequently in mussels adjacent to confined rival males (24 trials) than mussels without a male (12 trials), irrespective of the size of the territorial or rival male. This difference was significant (*G*-test, $G=4.08$, d.f.$=1$, $p=0.043$).

The rate of pre-oviposition ejaculation by territorial males into mussels with a rival male adjacent to it was significantly higher than without a rival (paired *t*-test, $t_{32}=2.27$, $p=0.030$). The mean±s.e. rate of pre-oviposition ejaculation with a rival was 13.2 ± 2.45 and without $5.7\pm1.51\times10\ \text{min}^{-1}$. The rate of aggression prior to spawning by the territorial male directed towards the rival male was significantly higher towards large than small rivals (unpaired *t*-test, square-root transformation, $t_{31}=2.30$, $p=0.028$; figure 2). However, there was no significant difference in the rate of aggression with respect to rival size immediately following spawning (unpaired *t*-test, square-root transformation, $t_{31}=0.09$, $p=0.930$; figure 2). There was no significant difference in the rate at which territorial males led females to mussels with or without a rival (Wilcoxon paired test, $T=89$, $n=33$, $p=0.223$). The mean±s.e. rate of leading to mussels with a rival was 2.3 ± 0.84 and without $3.1\pm0.76\times10\ \text{min}^{-1}$.

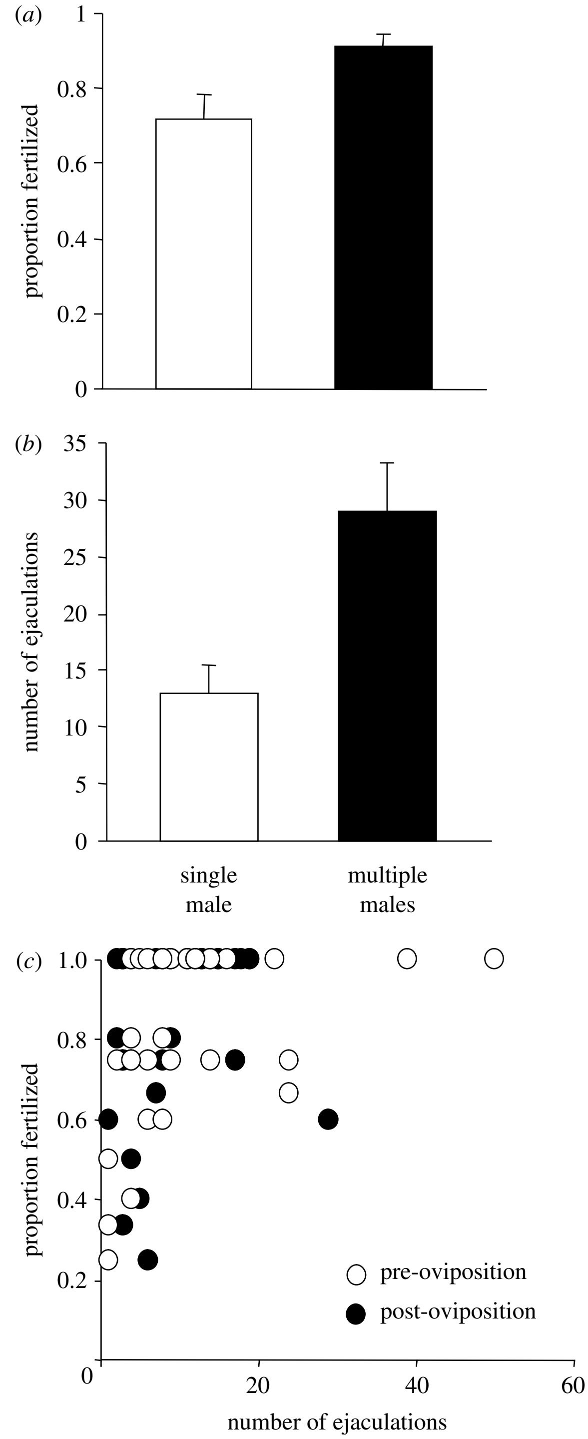

Figure 1. Ejaculation and fertilization success of male *R. sericeus*. (*a*) The mean proportion of eggs fertilized by single and multiple males. Error bars are one standard error. (*b*) Mean total ejaculations per spawning by single and multiple males. Error bars are one standard error. (*c*) Correlation between fertilization success and number of ejaculations pre- and post-oviposition.

Females inspected the exhalent siphons of mussels with a rival male adjacent at a significantly higher rate than without a male (Wilcoxon paired test, $T=403$, $n=33$, $p=0.002$; figure 3). In addition, females skimmed at a significantly higher rate over mussels with a rival male adjacent than without (Wilcoxon paired test, $T=229$, $n=33$, $p=0.025$; figure 3). The mean number of eggs deposited by females in mussels close to sneakers was greater if the rival male belonged to the large size class

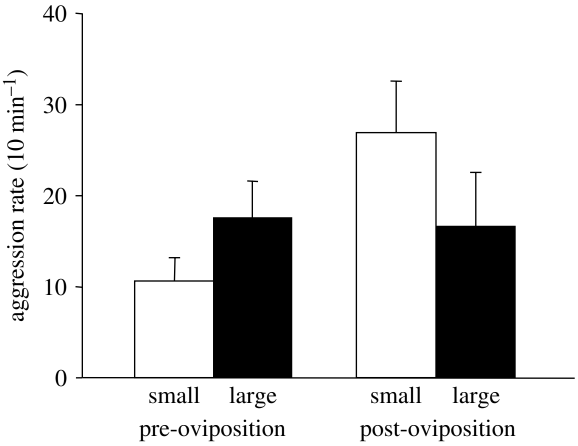

Figure 2. Mean rate of aggression by territorial male *R. sericeus* to rivals of two size classes. Left, pre-oviposition. Right, post-oviposition. Error bars are one standard error.

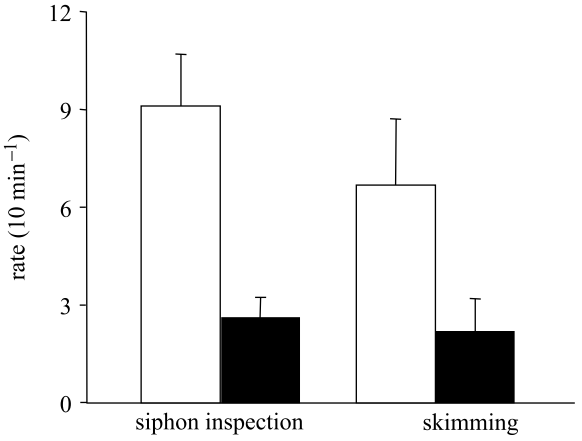

Figure 3. Female response to additional males in *R. sericeus*. Left, the mean rate of mussel siphon inspection with (white bar) and without an additional male (black bar). Right, the mean rate of skimming behaviour with (white bar) and without an additional male (black bar). Error bars are one standard error.

(unpaired *t*-test; $t_{28}=2.25$, $p=0.032$; figure 4*a*). After spawning, females remained within two body lengths of the mussel in which they had spawned for a longer period if the territorial male was small (unpaired *t*-test, log+1 transformation, $t_{31}=4.28$, $p<0.001$; figure 4*b*).

4. DISCUSSION

In this study we conducted two experiments to investigate whether the fertility of female bitterling is limited by fertilization success, and whether they engage in behaviour that may increase the probability of their eggs being fertilized.

In our first experiment we found that the proportion of fertile eggs increased significantly when more males participated in a spawning (figure 1*a*), illustrating that there may be substantial benefits to females of mating with more than one male. We do not have estimates of egg fertility from field studies. However, egg mortalities and ejections by mussels have proven common in field (Smith *et al.* 2000*b*) and mesocosm (Reichard *et al.* 2004*b*) studies by us, and may be associated with the failure of fertilization. The fact that the total number of ejaculations was higher in spawnings with a group of males (figure 1*b*), and the frequency of pre-oviposition ejaculations correlated positively with fertilization success (figure 1*c*) demonstrated that participation in a spawning by sneaking males increased the probability of fertilization. This effect may have arisen either through an increase in the volume of sperm released, or an increase in the probability of genetic compatibility between sperm and ova. Insurance of fertilization may be a particular problem for bitterling, given that the sperm must first pass through the mussel gill before reaching the eggs to fertilize them. However, sperm limitation has also been recorded in other fishes (Nakatsuru & Kramer 1982; Petersen *et al.* 2001), and may be a characteristic of reproduction in many taxa (Wedell *et al.* 2002). Notably, pre- rather than post-oviposition ejaculation correlated with fertilization success (figure 1*c*), supporting previous findings that pre-oviposition ejaculation relates most directly to male reproductive success in bitterling (Reichard *et al.* 2004*b*). Pre-oviposition sperm release occurs in other fish species (Sevenster-Bol 1962; Le Comber *et al.* 2004), and may be a widespread, but overlooked, feature of reproduction in fishes.

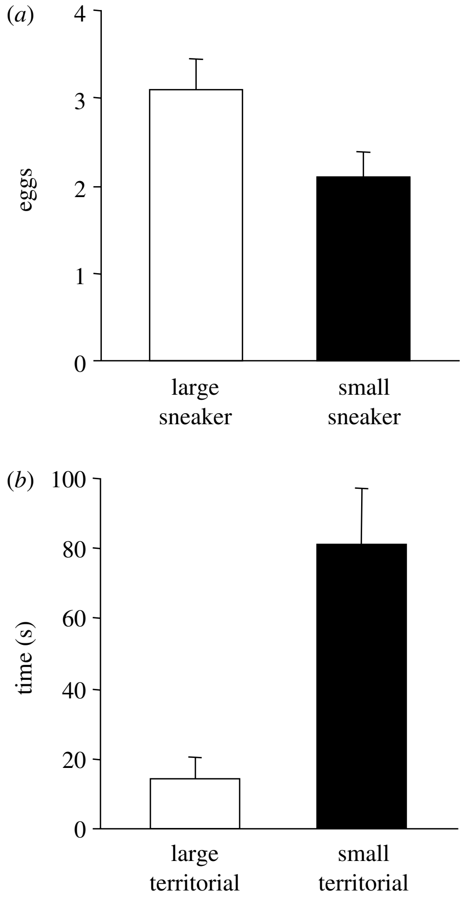

Figure 4. Effect of male size on female spawning response in *R. sericeus*. (*a*) Mean number of eggs deposited by females in mussels close to sneakers of two size classes. (*b*) Mean time to departure from a mussel by females following spawning with territorial males of two size classes. Error bars are one standard error.

In our second experiment females performed mussel inspection and skimming behaviour over mussels adjacent to a sneaker at significantly higher rates than over control mussels (figures 3 and 4). These two behaviours considerably increase the conspicuousness to males of females that are about to spawn, and may serve as cues to males that a spawning is imminent. Mussel inspection precedes most spawnings and probably serves in assessment of mussel quality (Smith *et al.* 2001). The function of skimming, which involves a female performing a spawning action but without inserting her ovipositor into the mussel gill chamber, is not understood, but is a further conspicuous cue, and possibly a signal, that a female may be about to spawn. In field and laboratory observations and experiments we have observed that skimming is often followed by the arrival of sneakers, and elicits sperm release by courting territorial males (Y. Zhu, unpublished data). Notably, females of the closely related *Rhodeus ocellatus* perform siphon inspections prior to pair spawnings but not during group spawnings, when up to 18 males participate in a spawning together (Kanoh 2000). This observation is consistent with our hypothesis that siphon inspection may serve to signal female spawning intent, with the signal redundant when a female spawns with a group of males and fertilization is assured. Female *R. ocellatus* also engage in skimming behaviour, as do several other bitterling species (Reichard & Smith, unpublished data), though there are no published studies yet that demonstrate an association between skimming and male mating tactics in these species.

We further found that females released significantly more eggs in spawnings with large rather than small bottled males (figure 4*a*). Mussel quality is an important determinant of female spawning decisions (Smith *et al.* 2000*b*). However, large males are more successful than small in attracting females for spawning, irrespective of mussel quality (Smith *et al.* 2002), indicating that sneaker quality may play a role in female spawning decisions. Fu *et al.* (2001) showed that female bluegill sunfish, *Lepomis macrochirus*, released more eggs in the presence of sneakers, and female bitterling may modulate egg number per spawning with respect to mussel quality (Mills & Reynolds 2002). Following spawning, females remained close to a mussel if the territorial male was small (figure 5*a*). Territorial males typically attempt to drive the female away from the mussel following spawning (Smith *et al.* 2004). The same behaviour is well described in nest-spawning fishes such as *Gasterosteus aculeatus* (Wootton 1976), though its function was hitherto thought to be that of preventing egg cannibalism by the female (Smith & Reay 1991). In the case of bitterling, egg cannibalism is impossible because the mussel protects the eggs. An alternative explanation is that the presence of a female with an extended ovipositor increases the chance of post-oviposition sneaking by rivals. Post-oviposition ejaculations may have lower fertilization success than pre-oviposition, but are still responsible for fertilizations (Reichard *et al.* 2004*a*). In our study, females reciprocated aggressive behaviour by the territorial male and actively resisted being driven away. The aggressive behaviour of the male following spawning, and the response of the female may reflect the conflicting adaptive outcomes in each sex to the risk of sperm competition following spawning.

In pair spawnings, the fertility of female bitterling appears to be limited by either sperm availability, or sperm and egg compatibility. In birds, the ubiquity of EPCs has been explained on the basis that there is a benefit to the female through controlling paternity, with intersexual conflict an inevitable outcome of such matings (Birkhead 1998). In bitterling, female behaviour that increases the number of males participating in a spawning is likely to enhance female fitness, by increasing fertilization success from an increased volume of sperm released per mating, or from an increased probability of genetic compatibility between sperm and ova. Thus, our results suggest a conflict between male assurance of paternity and female assurance of fertility. Male bitterling attempt to ensure their paternity by sperm loading (Smith *et al.* 2002) or sperm competition avoidance (Smith *et al.* 2003). Territorial male bitterling also engage in multiple ejaculations during pair spawnings, and this may be one way by which they are able to resolve this conflict; by releasing high numbers of sperm males may be able to induce females to spawn. However, this conflict also raises the possibility that males could manipulate female spawning behaviour, possibly by feigning ejaculations or modulating ejaculate size (Pateman-Jones, unpublished data).

We thank Luděk Bartoš, Paul Hart, Marcel Honza, Michal Janáč, Paulina Kaniewska, Steve Le Comber, Eamonn Mallon, Markéta Ondračková, Chris Pateman-Jones, Rowena Spence and Ashley Ward for assistance with fish collecting, laboratory experiments, and comments on the manuscript. Financial support for this study came from the Grant Agency of the Academy of Sciences of the Czech Republic, project 600930501. CS and MR designed and conducted the study. CS analysed the data, and CS and MR wrote the manuscript.

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