Carl Smith · Martin Reichard · Pavel Jurajda

Assessment of sperm competition by European bitterling, *Rhodeus sericeus*

Abstract We investigated male assessment of sperm competition in the bitterling, *Rhodeus sericeus*, a freshwater fish that spawns on the gills of living unionid mussels. Field experiments showed males increased their inspection rate of mussels into which a testis solution containing sperm had been experimentally released. Males avoided leading females to mussels that contained high numbers of embryos, but did not alter their leading behavior in response to the presence of sperm. In laboratory experiments males also increased their inspection rate of mussels into which a testis solution had been released and also failed to alter their leading behavior in response to the presence of sperm in mussels. However, males avoided leading females to mussels in close proximity to other males, and thereby may avoid sperm competition. In a second field study, territorial males were shown to ejaculate into mussels at a low rate in the absence of competitors, increase the frequency of ejaculations in competition with a rival, then decrease relative ejaculate expenditure as the number of competing males increased. Observed data were shown to be significantly correlated with predicted estimates of ejaculate expenditure for a model of sperm competition intensity. We discuss our results in the context of adaptive responses of males to sperm competition.

Keywords Alternative mating tactics · Acheilognathinae · ESS model · Sneaking · Strategic ejaculation

C. Smith (✉) · M. Reichard
School of Biological Sciences, Queen Mary University of London,
London, E1 4NS UK
e-mail: c.smith@qmul.ac.uk
Tel.: +44-20-78823056, Fax: +44-20-89830973

M. Reichard · P. Jurajda
Institute of Vertebrate Biology,
Academy of Sciences of the Czech Republic,
Květná 8, 603 65 Brno, Czech Republic

Introduction

Reproductive behavior can vary both within and between sexes (Gross 1996). Among male fishes a range of alternative mating tactics can be found, which typically involve males fertilizing the eggs of females that have been courted by rivals, thereby avoiding any costs of courtship. Alternative mating tactics have been considered as reproductive parasitism (Taborsky 1994), and are associated with sperm competition in species both with internal and external fertilization (Birkhead and Møller 1998).

Parker et al. (1996) made a distinction between sperm competition risk and sperm competition intensity; where sperm competition risk is the probability that ejaculates will overlap temporally or spatially during mating, whereas sperm competition intensity is the extent in overlap of ejaculates once sperm competition occurs (Simmons and Siva-Jothy 1998). Males are sensitive to sperm competition occurring at a mating, and often alter their reproductive behavior appropriately, engaging in what has been termed 'strategic ejaculation' (Martin and Hosken 2002). Modulation of ejaculate size in the face of sperm competition has been recorded across a broad range of taxa; including insects (Gage and Barnard 1996), crustaceans (Jivoff 1997), birds (Nicholls et al. 2001), and fishes (Marconato et al. 1995).

Among species, ejaculate expenditure on a mating is predicted to correlate positively with intensity of sperm competition (Parker 1982). However, the same pattern is not repeated within species. Parker et al. (1996) derived a series of sperm competition game theory models for a hypothetical fish species, which varied in the extent to which males are able to assess the number of competing males (the intensity of sperm competition), to explore the relationship between male assessment of sperm competition intensity and ejaculate expenditure. The models showed that the evolutionary stable strategy (ESS) for ejaculate effort should increase with the average sperm competition intensity for the species (or population), but that with improved information to assess the number of

competitors they face, males should reduce effort as the number of competitors present at a spawning increases above two. With no competitors at a mating, males are predicted to ejaculate relatively little sperm. A decrease in ejaculate expenditure with increased sperm competition intensity is expected because of the reduced relative gain in fitness with each unit of expenditure.

We investigated male assessment of sperm competition in the European bitterling, *Rhodeus sericeus*, a freshwater fish belonging to the sub-family Acheilognathinae, which have a unique spawning symbiosis with freshwater mussels. During the spawning season males develop bright nuptial coloration and defend territories around mussels. Female bitterling develop long ovipositors that they use to place their eggs onto the gills of a mussel through the mussels' exhalant siphon. Males fertilize the eggs by releasing sperm into the inhalant siphon of the mussel, so that water filtered by the mussel carries the sperm to the eggs. Developing embryos reside inside the mussel for approximately 1 month during which time they develop into actively swimming larvae. Bitterling display remarkable morphological, physiological and behavioral adaptations for using mussels as spawning sites, and they represent a valuable model in behavioral, population and evolutionary ecology. The value of the bitterling arises from it having a spawning site that can be easily manipulated and assessed for quality. Additionally, bitterling can be observed under natural conditions and they adapt readily to a laboratory environment (Wiepkema 1961; Smith et al. 2000a). Several mussel species are used by bitterling for spawning. Smith et al. (2000b) used field and laboratory experiments to show that female bitterling prefer spawning in *Anodonta anatina*, *Unio pictorum* and *U. tumidus* rather than in *A. cygnea*, and spawn at a lower rate in mussels containing high numbers of bitterling embryos. Mortality rates of bitterling embryos in mussels were strongly density dependent and the strength of density dependence varied among mussel species, with the strongest density-dependent mortality of embryos occurring in *A. cygnea*. The cause of embryo mortality in mussels, and the cue used by female bitterling to assess mussel suitability for oviposition, may be related to the oxygen conditions inside a mussel (Smith et al. 2001). In a further field study, Smith et al. (2002) showed females chose mussels on the basis of both the number of embryos already in a mussel and the body size and/or extent of red coloration of the eyes of the male guarding the mussel (male size and eye color were correlated). There was a significant interaction between the number of embryos in mussels and male size/ extent of red eye coloration, suggesting that direct and indirect benefits may both play a role in female oviposition choice.

Alternative mating tactics that may be associated with sperm competition are common in bitterling (Smith et al. 2002). These include males releasing sperm into the inhalant siphon of a mussel in which a pair has recently spawned. Also, both territorial males and non-territorial males perform pre-oviposition ejaculation into mussels

(Kanoh 1996, 2000; Smith et al. 2002), presumably to obtain precedence for their sperm in the event that a female spawns in a mussel. Thus, sperm competition may occur within the gill chamber of mussels. Territorial male bitterling display two adaptations for sperm competition. Males may avoid leading females to mussels in which rivals have already released sperm, an example of sperm competition avoidance. Males may also increase their rate of sperm release into mussels into which a rival male has released sperm, an example of sperm loading (Smith et al. 2002). Bitterling are ideal for quantifying sperm competition intensity because ejaculations can be easily recognized in both territorial males and competitors.

In an aquarium study of a naturalized population of *R. sericeus*, Candolin and Reynolds (2002a) observed the pattern of sperm release by males under competition. Prior to female oviposition, sperm releases matched the predictions of Parker et al.'s (1996) model of sperm expenditure, though the pattern of sperm release following oviposition failed to match predictions. Candolin and Reynolds (2002a) also attempted to quantify ejaculate size in *R. sericeus*, showing that ejaculate size did not vary among ejaculations from the same males at different levels of competition.

Here we use field and laboratory experiments to investigate the assessment of sperm competition by male bitterling. We first investigate the cues used by territorial male bitterling in sperm competition assessment. We then measure male ejaculate expenditure in response to variation in sperm competition intensity, and test these data against Parker et al.'s (1996) ESS sperm competition model.

Methods

Male oviposition behavior in relation
to mussel fullness with embryos and sperm presence

We conducted a field experiment to investigate male oviposition choice in relation to two factors: mussel fullness with embryos, and the presence of sperm in a mussel. We examined male response to mussel fullness, which is not related to sperm competition, because this is an important variable that is known to determine mussel 'quality' as a spawning site (Smith et al. 2000b, 2002). Thus we were able to measure the response of males to sperm presence in relation to mussel fullness with embryos. All field and laboratory data were collected between April and June 2001. Field studies were conducted in Lake Dědova in the southeast of the Czech Republic, at the center of the natural range of the bitterling in Europe. Visibility in this lake was good (Secchi disc reading of 1.0–1.3 m) and bitterling were abundant.

Two hundred *U. tumidus* were collected in mid-April, at the start of the bitterling spawning season, from a neighboring lake and transported to the test lake. *U. tumidus* were used because they were abundant and bitterling readily use them as spawning hosts. Mussels were randomly assigned to two treatments. Mussels in the 'low embryo' treatment were distributed among three plastic baskets that were half filled with sand and covered with netting that prevented bitterling from spawning in them, but allowed normal feeding and ventilation by the mussels. The remaining mussels were assigned to the 'high embryo' treatment and were distributed among another three baskets, but left exposed to allow

bitterling to spawn in them. The baskets were placed approximately 3 m from the bank in approximately 1 m of water.

Behavioral tests began 16 days after transferring the mussels to the test lake. Tests were conducted at 12 arenas around the lake margin 1–3 m from the shore and in a water depth of approximately 0.4–1.0 m. Four flowerpots filled with sand were placed at each arena and a single mussel was placed in each flowerpot both before behavioral tests began and throughout the experiment when behavioral observations were not taking place, to ensure a territorial male bitterling was always present.

Once a male had established a territory around a mussel in a flowerpot at one of the arenas, four test mussels were placed in a predetermined random order in each of the flowerpots. The four mussel treatments were; mussel with low number of embryos without sperm, mussel with high number of embryos without sperm, mussel with low number of embryos with sperm, and mussel with a high number of embryos with sperm. The sperm treatment was applied by a snorkeller immediately after the mussels were placed in position and had begun filtering water at a steady rate. A 0.5-ml sample of a testis solution was gently released into each mussel's inhalant siphon using a plastic 1-ml syringe. The testis solution was prepared by dissecting the testes from a subsample of sexually mature 1-year-old male bitterling collected using an electroshocker from a stream close to the test lake. Testes were dissected from males immediately after killing by cutting the spine at the base of the skull with a sharp pair of scissors. Each testis was cut in half, then frozen at –20°C in a 2-ml vial. The testes were defrosted when required, shaken with 1 ml of lake water, then the sperm suspension drawn into the syringe and used immediately. Examination of testis solutions at 400× magnification showed they comprised a high density of non-motile sperm (see below), with some fragments of testicular tissue. The solution may also have contained hormones, particularly androgens, though we did not test hormone levels of testis solutions.

A record was kept of the standard length (length from the tip of the snout to the base of the tail) of the male from which the testes were taken. As a control for mussels that did not receive the sperm treatment, 0.5 ml of lake water was gently released into each mussel's inhalant siphon from a syringe. To quantify the sperm released into mussels, counts of sperm in 12 testis solutions from 8 different males were made at 400× magnification using a hemocytometer. Sperm were counted in forty 0.25×0.25-mm cells from at least five subsamples from each testis solution to obtain a total sperm count for the solution.

A snorkeller observed the mussels from a distance of 1.0–1.5 m. Records were made of the rate at which territorial males inspected mussel siphons and led females to each mussel, for 8 min. Thus, leading rate was a continuous variable and none of the treatments were mutually exclusive. A period of 8 min exceeds the maximum period for which Kanoh (1996) found rose bitterling, *R. ocellatus*, sperm remained capable of fertilizing eggs after stripping from males. After 8 min, the location of each of the mussels under test was rearranged according to the next random pattern and a further 0.5 ml of testis solution was added to those mussels receiving the sperm treatment. Following a further 8 min the mussels in the arena were measured and marked to ensure they were not used again. A subsample of 12 mussels from the 'low embryos' and 'high embryos' groups were dissected to quantify their fullness with embryos. To avoid pseudoreplicating bitterling behavior in experiments we recorded distinguishing features, such as lost scales or the presence of external parasites of fish at each test arena. Data were recorded by three snorkellers in all arenas. After completion of the experiment, mussels were left in the lake until the end of the spawning season to ensure all juvenile bitterling were released.

Male oviposition behavior in relation to rival male proximity and sperm presence

Laboratory experiments were conducted at the Institute of Vertebrate Biology in Brno. An experiment was conducted to investigate male oviposition choice in relation to two factors: the presence of sperm in a mussel, and the close proximity of another male bitterling to a mussel. Sixty experimental fish were collected from a stream close to Lake Dědova using an electroshocker and transported in river water to the aquarium at the Institute of Vertebrate Biology. Fish were housed in eight aquaria measuring 75x40x40 cm (length × height × width) under natural day length and fed live bloodworm (*Tubifex* spp.) and dried fish flake food. Aquaria were aerated through an airstone and water quality was maintained using filters. Experiments began 8 days after the fish were collected. Unused mussels from the male oviposition behavior in relation to mussel fullness with embryos and sperm presence experiment were transported to the Institute of Vertebrate Biology and stored in flowerpots in a garden pond before use. During previous studies with bitterling over the past 8 years we have housed mussels in the pond without observing any detrimental effects; phytoplankton food was abundant, mussel siphons were always open, showing they filtered the water normally, and mussel survival was 100%.

Experiments were conducted in three aquaria measuring 75x40x40 cm with a 3-cm deep sand substrate. Mean water temperature during the experiment was 17.0 (0.12)°C (all means are given with the standard error in parentheses). A single male bitterling was placed in an experimental aquarium and allowed to establish a territory over a period of 24 h around a *U. tumidus* placed in a sand-filled flowerpot. After 24 h a female bitterling with an extended ovipositor was placed in the aquarium and the pair presented with two *U. tumidus* 12 cm apart, one containing sperm and one without sperm. The mussel already in the aquarium was removed for the duration of each test. A testis solution was applied to mussels as for the experiment on the effect of male oviposition behavior in relation to mussel fullness with embryos and sperm presence, with the same volume of sperm used and from the same source. Two clear plastic bottles, 28 cm high and 8 cm diameter, were placed 7 cm from each of the two mussels, with the two bottles 26 cm apart. A sexually mature male bitterling was placed in one bottle and a mature female, without an extended ovipositor, was placed in the other as a control. Territorial males were exposed to two randomly predetermined treatments; mussel with sperm + male versus mussel without sperm + non-spawning female, and the alternative treatment of mussel with sperm + non-spawning female versus mussel without sperm + male. Records were made of the rate at which the pair inspected mussel siphons, males led the female to mussels, ejaculated into mussels and the frequency of aggression (number of bites or butts) directed towards either the bottled male or female, for 8 min. After 8 min, the mussels and fish in bottles were rearranged according to the next random treatment, such that each experimental pair were exposed to both treatments in a random order, and a further 0.5 ml of testis solution was added to mussels receiving the sperm treatment. Following a further 8 min all the experimental subjects were removed and measured and none were used in the experiment again.

Ejaculate effort and sperm competition intensity

Field observations were conducted during May 1999 and 2000 in Lake Dědova. Results from 2000 on female spawning rates are presented in Smith et al. (2002), though no results are repeated here. A series of sand-filled flowerpots were positioned at approximately 20-m intervals around the lake margin and a single *A. anatina*, collected from a neighboring lake, placed in each. Once a male bitterling had established a territory in an arena, the mussel was replaced with a single *A. anatina* that had not received any spawnings. A snorkeller then observed the mussel until a female spawned in it. A record was kept of the frequency that the territorial males ejaculated into the inhalant siphon of the mussel, the frequency that rival males ejaculated into the mussel, the number of rival males that ejaculated into the mussel, and the rate of aggressive behavior directed by the territorial male towards rival males. Individual males were recognized by recording unique features of their appearance during spawning. Individual characteristics of bitterling can be readily recorded under natural conditions

and used to separate spawning individuals (e.g., Smith et al. 2000b, 2001, 2002).

After a spawning was completed (including post-oviposition ejaculations), the territorial male, spawning female and rival males were captured in a cylindrical pop net set around each flowerpot. Each fish was measured to the nearest 1 mm then marked by fin-clipping. The same territorial male, female or mussel was not used in any further tests. Not all rival males could always be captured and some may have been involved in further tests, but always with different territorial males, females and mussels.

Data analyses

Data for male ejaculation rate and number of rival males that ejaculated into mussels were fitted to the sperm competition model of Parker et al. (1996) for males with perfect information about competitors:

$$e_{ESS} = \left[\frac{(N_i - 1)}{N_i^2} \right] N.$$

where e_{ESS}=ESS ejaculate expenditure (as a relative measure of the average total reproductive effort per spawning), N_i=number of competitors present at a spawning of type i, which occur with probability $q(N_i)=qi$, and N=mean number of competitors at a spawning for the study population. Since estimates of total reproductive expenditure per spawning are not available for bitterling, we estimated e_{ESS} as a function of the maximum number of ejaculates per spawning for males in the study population. As a consequence of using this function for ejaculate expenditure, our estimates of observed e_{ESS} cannot be compared directly with predicted estimates from Parker et al.'s (1996) model. Model predictions of territorial male e_{ESS} were compared with observed estimates of e_{ESS} using Pearson's correlation. We also tested the fit of the model using total competitor ejaculations, rather than number of competing males, using a Spearman's rank correlation for non-parametric data.

The mean number of competitors at a spawning (N), and the maximum male ejaculation rate was estimated from Smith et al. (2000b) for the study population. For $N_i=1$, when there is no sperm competition, the minimum ejaculation expenditure was estimated from the mean ejaculation rate for a spawning in the absence of competitors from Smith et al. (2000b). Data analyses were carried out using MINITAB.

Results

Male oviposition behavior in relation to mussel fullness with embryos and sperm presence

A total of 40 choice tests using 80 mussels was completed with 20 different males. As anticipated, the number of bitterling embryos on the gills of mussels in the 'high embryo' group subsample was significantly higher than the 'low embryo' group (unpaired two-tailed t-test, data square-root transformed $t=6.95$, $df=10$, $P<0.001$). The mean number of embryos in the 'high embryo' group was 23.5 (4.84), in the 'low embryo' group 0.8 (0.48). There was no significant difference in the mean length of mussels assigned to the four treatments (one-way ANOVA $F=0.90$, $df=3,48$, $P=0.446$).

The rate of inspection of mussels by territorial males was significantly higher if testis solution had been released into the mussel's inhalant siphon (balanced 2-way ANOVA, data square-root transformed $F=4.09$, $df=1,76$, $P=0.047$; Fig. 1). However, there was no significant
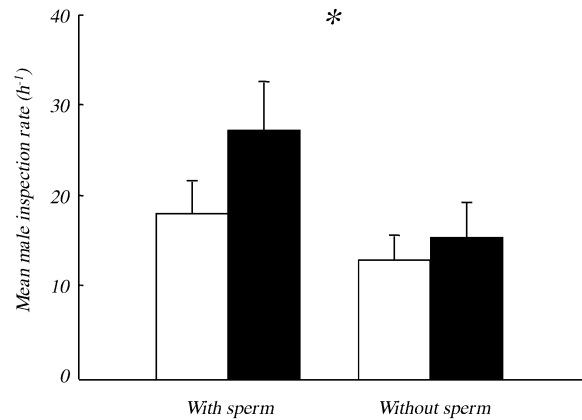

Fig. 1 The mean rate of inspection of the inhalant siphons of mussels by territorial male bitterling (h^{-1}) with high (*white bars*) and low (*black bars*) numbers of embryos in mussels and with and without the addition of a sperm treatment to mussels. *Error bars* are 1 SE and an *asterisk* signifies significant difference at $\alpha=0.05$
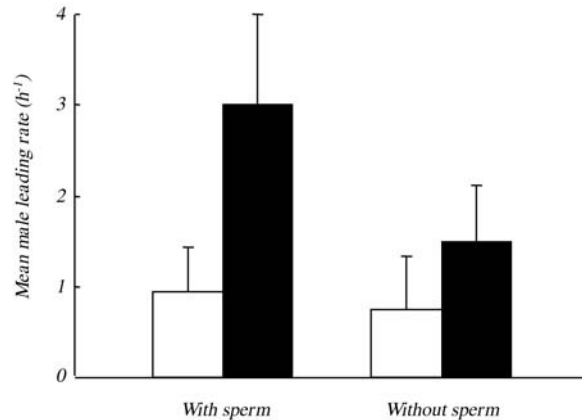

Fig. 2 The mean rate of leading females to mussels by territorial male bitterling (h^{-1}) with high (*white bars*) and low (*black bars*) numbers of embryos in mussels with and without the addition of a sperm treatment to mussels. *Error bars* are 1 SE

effect of mussel fullness with embryos on inspection rate ($F=3.5$, $df=1,76$, $P=0.350$), and there was no significant interaction between the variables ($F=0.55$, $df=1,76$, $P=0.461$). The presence of sperm had no effect on the rate at which males led females to mussels (Scheirer-Ray-Hare test cumulative $\chi^2=0.78$, $df=1$, $P=0.210$; Fig. 2). There was an effect of mussel fullness with embryos on leading rate (cumulative $\chi^2=0.96$, $df=1$, $P=0.041$), with males leading females to mussels in the 'low embryo' group at a higher rate regardless of sperm treatment. There was no interaction between the variables (cumulative $\chi^2=0.69$, $df=1$, $P=0.306$). There was no effect of either sperm (Scheirer-Ray-Hare test: cumulative $\chi^2=0.79$, $df=1$, $P=0.210$), or mussel fullness on male ejaculation rate into mussels (cumulative $\chi^2=0.91$, $df=1$, $P=0.095$), nor was there an interaction between these treatments (cumulative $\chi^2=0.79$, $df=1$, $P=0.210$).
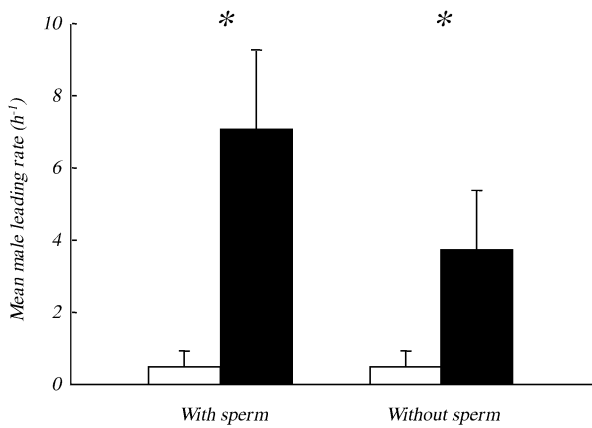
Fig. 3 The mean rate of leading females to mussels by territorial male bitterling (h^{-1}) with (*white bars*) and without (*black bars*) a male in close proximity to mussels, with and without the addition of a sperm treatment to mussels. *Error bars* are 1 SE and *asterisks* signify significant differences at $\alpha=0.05$

Fig. 4 The mean rate of inspection of the inhalant siphons of mussels by female bitterling (h^{-1}) with high (*white bars*) and low (*black bars*) numbers of embryos in mussels and with and without the addition of a sperm treatment to mussels. *Error bars* are 1 SE and an *asterisk* signifies significant difference at $\alpha=0.05$

Female inspection rate of mussel siphons did not differ with sperm treatment (Scheirer-Ray-Hare test: cumulative $\chi^2=0.56$, $df=1$, $P=0.440$), or with mussel fullness with embryos (cumulative $\chi^2=0.72$, $df=1$, $P=0.280$). There was also no interaction between the variables (cumulative $\chi^2=0.56$, $df=1$, $P=0.441$).

The total number of sperm in each 0.5 ml sample of testis solution released into mussels was 9.4 (2.32)x10^6. There was no relationship between sperm number in samples and the standard length of males from which testes were taken (Pearson's correlation, $\log_{10}$ transformation $r=0.51$, $df=7$, $P=0.194$).

Male oviposition behavior in relation to rival male proximity and sperm presence

A total of 18 different males was tested using 72 mussels. The aggression rate of territorial males towards bottled males was significantly higher than towards bottled females [23.7 (2.48) attacks h^{-1}, vs. 5.8 (1.50) attacks h^{-1}; unpaired two-tailed-test, square-root transformation $t=3.34$, $df=16$, $P=0.004$]. The rate of mussel inspection by territorial males was significantly higher if bitterling sperm had been released into the mussel's inhalant siphon (balanced two-way ANOVA, square-root transformation $F=5.90$, $df=1,68$, $P=0.018$). However, there was no significant effect of the presence of another male on inspection rate ($F=3.06$, $df=1,68$, $P=0.085$), and there was no significant interaction between the variables ($F=0.64$, $df=1,68$, $P=0.426$). Males did not differ in the rate at which they led females to mussels if sperm had been released into the mussel (balanced two-way ANOVA $F=1.46$, $df=1,68$, $P=0.231$). However, there was a significant effect of the presence of another male on leading rate ($F=11.16$, $df=1,68$, $P=0.001$; Fig. 3), with territorial males leading females to mussels without a male at a higher rate. There was no interaction between
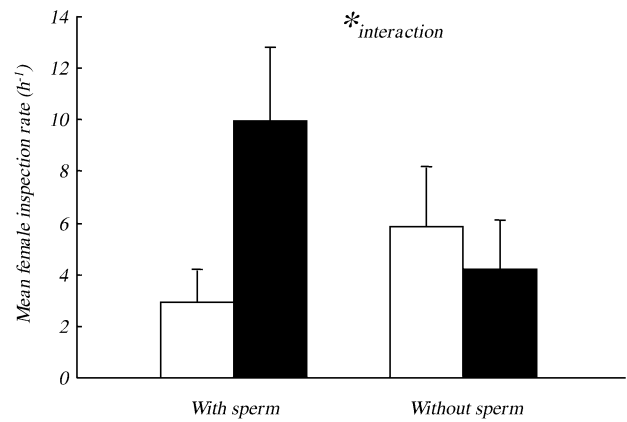
the variables ($F=1.46$, $df=1,68$, $P=0.231$). There was no effect of either sperm (balanced two-way ANOVA $F=0.33$, $df=1,68$, $P=0.566$), or the presence of a bottled male on territorial male ejaculation rate into mussels ($F=0.00$, $df=1,68$, $P=1.000$). Similarly, there was no effect of sperm (balanced two-way ANOVA $F=0.46$, $df=1,68$, $P=0.498$), or the presence of a bottled male on female inspection rate of mussels ($F=1.60$, $df=1,68$, $P=0.210$). However, there was a significant interaction between these variables ($F=4.17$, $df=1,68$, $P=0.045$; Fig. 4).

Bottled male fish did not respond to territorial males, but displayed low level courtship behavior to females by extending their fins. Bottled females did not respond to territorial males or to females. Bottled fish swam naturally and did not show signs of distress during the short time they were confined.

Ejaculate effort and sperm competition intensity

The estimated mean number of competitors at a spawning for the study population (N) was 1.6 (0.32) males (data from Smith et al. 2000b). The maximum male ejaculation rate for the study population was 15 ejaculations per spawning, and the mean rate in the absence of competitors (when $N_i=1$) 1.7 (0.09) ejaculations per spawning (Smith et al. 2000b).

Our test of Parker et al.'s (1996) model showed that territorial male bitterling responses to changes in sperm competition intensity largely conform with the predictions of the model. Ejaculate expenditure in the absence of competitors was low, representing 11% of the maximum. Ejaculation expenditure was greatest with a single competitor, and tended to decline with an increase in the number of competing males (Fig. 5). There was a significant correlation between observed and predicted e_{ESS} (Pearson's correlation $r=0.72$, $df=7$, $P=0.028$). However,
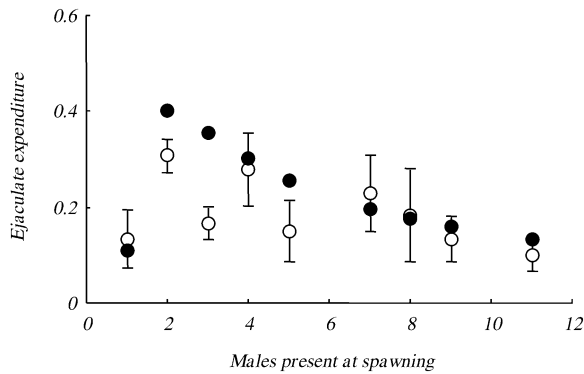
Fig. 5 Ejaculate expenditure (as a proportion of total reproductive effort per spawning) against the number of males competing at a spawning. Points are for predicted (*black points*) and observed (*white points*) ejaculate expenditure. *Error bars* are 1 SE
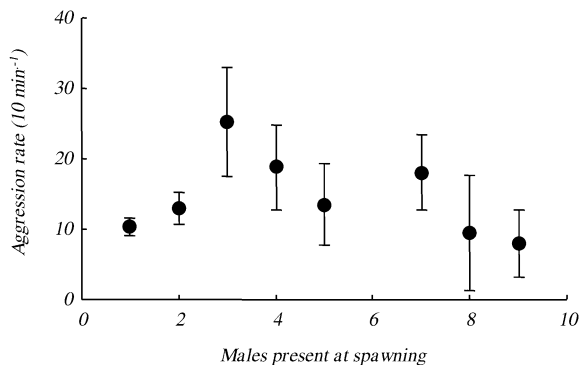

Fig. 6 Territorial male aggression rate towards competing males (10 min.$^{-1}$) against the number of males competing at a spawning. *Error bars* are 1 SE

there was no significant correlation if competing male ejaculation rate was used as the independent variable (Spearman's rank correlation r_s=0.12, *df*=7, *P*=0.513). Mean territorial male aggression rate towards competing males showed a distinctive pattern. There was a low level of aggression when just one or two males were present at a spawning, increasing to a maximum when three males were present. Further increases in the numbers of competing males resulted in a decline in aggression rate (Fig. 6).

Discussion

We used a field and laboratory experiment to investigate the effect of mussel fullness with embryos, the presence of sperm in the inhalant siphon of a mussel, and the presence of a potential rival on the oviposition choice of territorial male bitterling. In both experiments, territorial males increased the rate at which they inspected mussel siphons into which a testis solution had been released in comparison with mussels into which water was released. However, in neither case did males modify their leading

behavior in response to the presence of this treatment. Thus, males appeared to detect the sperm treatment, but did not alter their spawning behavior in response to it. Males may have increased mussel inspection rates in the sperm treatment in response to the taste or smell of sperm, testicular tissue or both in the mussel siphon.

Territorial males responded to mussel fullness with embryos, a measure of mussel quality as a spawning site, by reducing their rate of leading females to mussels containing high numbers of embryos. This behavior may be adaptive, though unrelated to sperm competition avoidance, since it would minimize the density-dependent mortality of embryos in mussels (Smith et al. 2000b). Territorial males also avoided leading females to mussels in close proximity to a rival male. This behavior may also be adaptive for a male if there is a risk that the rival male may attempt to fertilize eggs released during a spawning. There is good evidence, from both studies with rose bitterling (Kanoh 1996, 2000) and our own research with *R. sericeus* (Smith et al. 2001, 2002), that parasitized matings are both widespread and successful in bitterling. Further, sperm of the rose bitterling remains capable of fertilizing an egg for between 3 and 4 min after release (Kanoh 1996), and this may also be the case with *R. sericeus*. Thus, our findings imply that territorial male bitterling, while not responding to the presence of a rival's sperm in a mussel, may be sensitive to the proximity of a rival to potential oviposition sites, with males avoiding using mussels in which other males may have already released, or be about to release sperm. Our study does not identify the duration of the effect of a rival on the propensity of a territorial male to lead a female to a mussel. In neither our field nor laboratory experiment did we observe an increase in ejaculation rate by territorial males in response to either the sperm treatment or the presence of a competing male. This result appears to contradict Smith et al. (2002) who recorded sperm loading by males in response to rival male ejaculations. One possibility is that males respond with sperm loading to specific visual cues, such as rival males ejaculating into a mussel, which were not included in the experimental design. Ejaculation by male bitterling is clearly distinguishable to human observers and likely to be equally clear to other bitterling. Thus, while sperm competition avoidance appears to be elicited by the close proximity of a rival male to a mussel, sperm loading may be elicited only by the ejaculation behavior of a competing male.

For practical and animal welfare reasons it was impossible to test the responses of bitterling to naturally released fresh sperm. We tested the effect of frozen-thawed testis solution and fresh sperm and confirmed that mussels did not respond differently to these two in terms of remaining open for bitterling to inspect them or spawn in them (data not shown). However, this result implies nothing about the response of bitterling to frozen-thawed testis solution in comparison with fresh sperm. Freezing sperm, including cryopreservation, affects sperm motility in fishes (e.g., Ciereszko et al. 1996). However, frozen-thawed sperm retains other properties and resembles

fresh sperm closely (e.g., Viveiros et al. 2000). Thus, if bitterling respond to chemical cues in the sperm then frozen-thawed testis solution will be an adequate substitute for fresh sperm. Direct examination of the testis solutions showed they comprised principally sperm in suspension. Consequently, we believe this experiment represented a valid test of whether male bitterling respond to chemical cues from sperm in assessing the risk of sperm competition.

Female bitterling showed no difference in mussel inspection rates in response to sperm and male treatments. However, there was a significant interaction in female inspection rates to both these (Fig. 4). Females inspected mussels with a bottled male at a high rate and without a male but with sperm at a high rate. Conversely, females inspected mussels at a low rate if both a bottled male and sperm were present and if neither a male nor sperm were present. Females always inspect mussels before spawning in them, and mussel inspection probably provides information about the suitability of a mussel for spawning (Smith et al. 2001). In addition to providing information about mussel fullness with embryos, inspection may demonstrate to a female whether sperm is present in the gill chamber of a mussel, thereby ensuring fertilization of her eggs, or may even reveal that several males have released sperm into a mussel, thereby ensuring multiple paternity of her offspring. The implications of this significant interaction are not clear and warrant further investigation.

Observed ejaculate expenditure showed a significant correlation with the predictions of Parker et al.'s (1996) model. In our field study territorial males ejaculated into mussels at a low rate in the absence of competitors, increased the frequency of ejaculation in competition with a rival, then decreased relative ejaculation expenditure as the number of competing males increased. This result also fits with an earlier observation, reported by Smith et al. (2002), that males compete with solitary rivals by sperm loading, but will not attempt sperm loading, and even abandon defense of a mussel, in competition with large (up to 50) groups of males. A central assumption of Parker et al.'s (1996) model is that ejaculate size does not vary among ejaculations in relation to the risk of sperm competition. This assumption appears justified, since Candolin and Reynolds (2002a) found that ejaculate size did not vary among ejaculations in *R. sericeus* at different levels of competition from rival males. Substituting total ejaculate frequency as the independent variable in Parker et al.'s (1996) model failed to show a significant correlation between observed and expected ejaculate expenditure. This result further reinforces our experimental result that males do not use the presence or volume of sperm in assessing sperm competition intensity, but rather the number of competing males ejaculating into a mussel. It is notable that variance in observed ejaculate expenditure tended to increase with an increasing number of competing males (Fig. 5). This correlation of variance in male response with number of competitors may be because territorial males are less accurate in as-

sessing the number of competing males at high competitor abundance. Alternatively, or in addition, males may lack the cognitive ability to count high numbers of competitors.

Candolin and Reynolds (2002a) conducted an aquarium study with *R. sericeus*, in which they recorded ejaculation rates of males facing competition from varying numbers of rival males. They detected a similar pattern of ejaculations by territorial males to our own for pre-oviposition ejaculations, with ejaculation rate declining with greater competitor abundance. Following oviposition, however, sperm releases did not match the predictions of the model, though males did increase their aggression. Candolin and Reynolds (2002a) concluded that sperm release and aggression serve as alternative responses to sperm competition. Candolin and Reynolds (2002b) further described an unexpected "tolerance" by territorial male bitterling of rival males during the same study, though this behavior has never been reported under natural conditions. The unusual behavior observed by Candolin and Reynolds (2002a, 2002b) may be an artifact of their aquarium study, or a peculiarity of the naturalized population they studied.

The aggressive response of males to competitors in the present study showed a comparable pattern to that of ejaculate expenditure (Fig. 6), matching the observations of Candolin and Reynolds (2002a). Aggression rates were low in cases where no males ejaculated into mussels (though rival males were present), but increased to a maximum with two competitors, then declined with increasing numbers of competing males. Aggression represents an additional tactic for territorial males to counter reproductive parasitism, and represents a further expenditure by males on a spawning. Non-territorial males do not reciprocate the aggression of territorial males, and usually avoid encounters with the territory holder. Smith et al. (2002) showed that small territory holding males were subjected to higher rates of reproductive parasitism than larger males, principally because they lacked the ability to aggressively defend mussels. Thus a combination of aggression and modulation of ejaculate expenditure appear to be the principal mechanisms by which territorial male bitterling counter sperm competition from males engaging in alternative mating tactics.

Parker et al.'s (1996) model is underpinned by a number of assumptions; fertilization is assumed to be instantaneous, ejaculate size to be constant, and sperm quality to not vary among males. The validity of these assumptions have not been fully investigated for bitterling, but future work will explore the reproductive success of male mating tactics using DNA microsatellites, measures of sperm volume and quality, and the dynamics of fertilization within the mussel gill.

In conclusion, the results provide support for Parker et al.'s (1996) model for sperm competition intensity, with male bitterling modulating ejaculation frequency in relation to competitor abundance. Male bitterling appear to use the close proximity of rivals to a mussel as the proximate cue for assessing sperm competition intensity.

We found no evidence that males are able to detect the sperm of rivals in the siphons of mussels. Males were shown to vary their aggressive behavior in relation to competitor abundance, and a combination of aggression and strategic ejaculation appear to be the principal mechanisms by which territorial male bitterling counter sperm competition.

References

Birkhead TR, Møller AP (1998) Sperm competition, sexual selection and different routes to fitness. In: Birkhead TR, AP Møller (eds) Sperm competition and sexual selection. Academic Press, London, pp 757–781

Candolin U, Reynolds JD (2002a) Adjustments of ejaculation rates in response to risk of sperm competition in a fish, the bitterling (Rhodeus sericeus). Proc R Soc Lond Ser B 269:1549–1553

Candolin U, Reynolds JD (2002b) Why do males tolerate sneakers? Tests with the European bitterling, Rhodeus sericeus. Behav Ecol Sociobiol 51:146–152

Ciereszko A, Toth GP, Christ SA, Dabrowski K (1996) Effect of cryopreservation and theophylline on motility characteristics of lake sturgeon (Acipenser fulvescens) spermatozoa. Theriogenology 45:665–672

Gage AR, Barnard CJ (1996) Male crickets increase sperm number in relation to competition and female size. Behav Ecol Sociobiol 38:227–237

Gross MR (1996) Alternative reproductive strategies and tactics: diversity within sexes. Trends Ecol Evol 11:92–97

Jivoff P (1997) The relative roles of predation and sperm competition on the duration of the post-copulatory association between the sexes in the blue crab, Callinectes sapidus. Behav Ecol Sociobiol 40:175–185

Kanoh Y (1996) Pre-oviposition ejaculation in externally fertilizing fish: how sneaker male rose bitterlings contrive to mate. Ethology 102:883–899

Kanoh Y (2000) Reproductive success associated with territoriality, sneaking and grouping in male rose bitterlings, Rhodeus ocellatus (Pisces: Cyprinidae). Environ Biol Fish 57:143–154

Marconato A, Tessari V, Marin G (1995) The mating system of Xyrichthys novacula: sperm economy and fertilization success. J Fish Biol 47:292–301

Martin OY, Hosken DJ (2002) Strategic ejaculation in the common dung fly Sepsis cynipsea. Anim Behav 63:541–546

Nicholls E.H, Burke T, Birkhead TR (2001) Ejaculate allocation by male sand martins, Riparia riparia. Proc R Soc Lond Ser B 268:1265–1270

Parker GA (1982) Why are there so many tiny sperm? Sperm competition and the maintenance of two sexes. J Theor Biol 96:281–294

Parker GA, Ball MA, Stockley P, Gage MJG (1996) Sperm competition games: individual assessment of sperm competition intensity by group spawners. Proc R Soc Lond Ser B 263:1291–1297

Simmons LW, Siva-Jothy MT (1998) Sperm competition in insects: mechanisms and the potential for selection. In: Birkhead TR, AP Møller (eds) Sperm competition and sexual selection. Academic Press, London, pp 341–434

Smith C, Reynolds JD, Sutherland WJ (2000a) Population consequences of reproductive decisions. Proc R Soc Lond Ser B 267:1327–1334

Smith C, Reynolds JD, Sutherland WJ, Jurajda P (2000b). Adaptive host choice and avoidance of superparasitism in the spawning decisions of bitterling (Rhodeus sericeus). Behav Ecol Sociobiol 48:29–35

Smith C, Rippon K, Douglas A, Jurajda P (2001) A proximate cue for oviposition site choice in the bitterling (Rhodeus sericeus). Freshw Biol 46:903–911

Smith C, Douglas A, Jurajda P (2002) Sexual conflict, sexual selection, and sperm competition in the spawning decisions of bitterling (Rhodeus sericeus). Behav Ecol Sociobiol 51:433–439

Taborsky M (1994) Sneakers, satellites, and helpers: parasitic and cooperative behaviour in fish reproduction. Adv Study Behav 23:1–100

Viveiros ATM, So N, Komen J (2000) Sperm cryopreservation of African catfish, Clarias gariepinus: cryoprotectants, freezing rates and sperm: egg dilution ratio. Theriogenology 54:1395–1408

Wiepkema PR (1961) An ethological analysis of the reproductive behaviour of the bitterling (Rhodeus amarus Bloch). Arch Neerl Zool 14:103–199s