Sperm depletion as a consequence of increased sperm competition risk in the European bitterling, *Rhodeus amarus*

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Sperm depletion occurs when males are unable to maintain ejaculate size over successive ejaculations and appears to be widespread across multiple taxa. We used the European bitterling, a fish that lays its eggs in the gills of freshwater mussels, in experiments to investigate sperm depletion over different temporal scales at different levels of sperm competition risk. The spermatozoa density of territorial male ejaculates declined over five consecutive ejaculations, but mean ejaculate size was significantly larger if the male was exposed to a rival, suggesting males were sensitive to sperm competition risk and modulated ejaculate size in accordance with theoretical models. However, following successive matings over the course of a day, males exposed to rivals produced significantly smaller ejaculates than males with a low risk of sperm competition. Results are discussed with reference to a possible intersexual conflict over the size and distribution of ejaculates in the context of male sperm depletion and female sperm limitation.

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In species in which females mate promiscuously, the ejaculates of different males may compete simultaneously to fertilize the same set of ova in a process termed sperm competition. Sperm competition is recognized as an important mechanism of sexual selection that has shaped the evolution of animal mating systems (Parker 1990, 1998; Andersson 1994; Simmons 2001). Where the probability of female promiscuity is high (a high risk of sperm competition) males often show adaptations for sperm competition, including adaptations of testis size and structure (Byrne et al. 2002), ejaculate size (Briskie 1993), sperm morphology (Snook 2005) and behavioural adaptations for sperm competition avoidance (Smith et al. 2002). Because ejaculates may be physiologically expensive to produce (Nakatsu & Kramer 1982; Van Voorhies 1992; Shapiro et al. 1994; Olsson et al. 1997; Warner 1997; Preston et al. 2001; Wedell et al. 2002), males are predicted to increase ejaculate expenditure when competing with a single rival, but reduce expenditure if the intensity of sperm competition (the extent of overlap of competing male ejaculates) is high, since the probability of fertilizing eggs diminishes with the number of competing males that attempt to fertilize the same set of ova (Parker et al. 1996). Males from a broad range of taxa appear sensitive to sperm competition and there is evidence that males may tailor ejaculate expenditure to maximize their long-term reproductive success (Gage 1991; Evans & Magurran 2000; Pilastro et al. 2002; Pizzari et al. 2003; Harris & Moore 2005; delBarco-Trillo & Ferkin 2006).

Sperm depletion or, conversely, the prudent control of ejaculate expenditure by males, also termed ‘sperm economy’, can occur when the allocation of sperm to current mating opportunities is traded off against the ejaculates of future matings. Sperm depletion can have consequences for female reproductive success, possibly resulting in females becoming sperm limited. Infertility rates in
nature can be high (Eberhard 1996), and females may sometimes obtain insufficient sperm to fertilize all their ova (Pitnick 1993; Royer & McNeil 1993; Stockley 1997; Rondeau & Sainte-Marie 2001; García-González 2004). In the blue-head wrasse, Thalassoma bifasciatum, an externally fertilizing fish, females prefer dominant males as mates but suffer reduced fertility when mating with these males as they produce smaller ejaculates than subordinate males (Shapiro et al. 1994; Rasotto & Shapiro 1998). The same can occur in lekking species, with females risking reduced fecundity when mating with dominant males (Jones 2001; Saether et al. 2001). Sperm limitation may lead to interfemale competition for access to males (Saether et al. 2001) and the evolution of male mate choice (Preston et al. 2005). There is also evidence that females may solicit matings from males, and mate with multiple partners, to increase their fertility (Gray 1997; Jennions & Petrie 2000; Smith & Reichard 2005; Reichard et al. 2007). Males may be especially susceptible to sperm depletion when they engage in sperm competition. If males increase ejaculate expenditure in response to an elevated risk of sperm competition they may face a concomitant increase in sperm depletion (Wedell et al. 2002).

In the present study we investigated the effect of sperm competition risk on male sperm depletion over successive ejaculations during a single mating, and over a series of matings, in the European bitterling, a small freshwater fish belonging to the carp family. The response of territorial male European bitterling to sperm competition appears consistent with that predicted by theoretical models of sperm competition. Territorial males elevate their ejaculation rate when competing with a rival (Candolin & Reynolds 2002; Smith et al. 2003), although there is no published evidence that they modulate ejaculate size. In addition, the fertility of bitterling eggs may not always be 100%. With only a single male to fertilize a clutch of eggs the rate of fertility averaged just 74%, rising to 94% when three males participated in a spawning (Smith & Reichard 2005).

During the spawning season, male bitterling either aggressively defend territories around mussels or perform sneaking behaviour, although males can play both roles and there are no morphological differences between territory holders and sneakers (Candolin & Reynolds 2002; Smith et al. 2002, 2004; Reichard et al. 2005, 2008). Dominance among males is determined by relative size (Casalini et al. 2009). Females develop long ovipositors, which they use to place their eggs into the gills of a mussel. Males fertilize the eggs by releasing sperm over the inhalant siphon of the mussel, often repeatedly in the course of a single mating. Water filtered by the mussel carries the sperm to the eggs where they are fertilized and undergo development for 3–4 weeks. Spawning occurs throughout the daylight hours, from dawn until sunset, and there is no evidence for temporal variation in spawning activity. Attempts to sneak fertilizations, by ejaculating over the mussels of rival males, are frequent (Smith et al. 2002) and successful (Reichard et al. 2004a, b). The reproductive biology of R. amarus was reviewed by Smith et al. (2004).

We measured ejaculate size by estimating the number of spermatozoa released during experimental spawnings to test two predictions. First, in competition with a single rival, territorial males should respond to a higher sperm competition risk by increasing their ejaculate expenditure, measured as size of ejaculate rather than simply ejaculate frequency. Second, as a consequence of increased ejaculation expenditure in response to greater sperm competition risk, males were predicted to face a higher probability of sperm depletion over a series of matings than males that did not face sperm competition from rivals. To enable these results to be interpreted in relation to typical rates of ejaculation, we conducted a field study to record male ejaculation rates under natural conditions.
was recorded and a cylindrical (diameter 80 cm) net set around the focal mussel was gently raised to capture all participants in the spawning. Fish retained in the net were captured using a hand net and marked by clipping the edge of their tail fin. Marked fish were immediately released, but the behaviour of marked territorial males was not recorded again. Fin clipping was accomplished quickly and thereby minimized any negative impacts on the fish, but unambiguously indicated a fish had been used in a trial. The amount of fin tissue removed was approximately 2% of the total fin area and fish did not show any obvious responses to marking; marked fish were frequently seen within a short time of marking (10–15 min) defending mussels and performing courtship and spawning behaviour. Overall, fin clipping was judged to be less invasive than tagging, but more dependable than reliance on natural variation in appearance. Mussels were replaced after a spawning occurred and were used only once in the study.

Estimating Ejaculate Size

To estimate the size of ejaculates, we attached a BD Venflon winged cannula (Becton Dickinson Infusion Therapy, Helsingborg, Sweden) to the shell of U. tumidus mussels. The cannula wings were glued to the mussel shell, so that the sheath of the cannula lay along the shell, with the tip resting immediately adjacent to the inhalant siphon at the point at which male bitterling release sperm over the mussel. A 1 m long piece of plastic tubing 5 mm in diameter was fixed to each cannula and a syringe fitted to the open end. Immediately following ejaculation by a male bitterling, 300 mm$^3$ of water was collected at the inhalant siphon of the mussel by drawing water into the plastic tubing using the syringe. The plastic tubing was detached at the junction with the cannula and the water sample was released into a 1 ml Eppendorf. The sample was gently mixed then pipetted onto a haemocytometer (Neubauer improved, VWR International, Vienna, Austria). We counted the spermatozoa in the sample using a binocular microscope (Nikon Eclipse E200) with a 40× objective. Sperm cells were counted in five 1 × 1 × 0.1 mm squares to obtain a mean sperm density for the subsample and multiplied up to the entire sample volume of 300 mm$^3$ to give an estimate of total ejaculate size. A volume of 300 mm$^3$ of water was sufficient to collect the entire ejaculate released over the mussel inhalant siphon, since collecting a larger volume did not yield higher estimates of total ejaculate size. No detrimental effects of attaching cannulas to mussels were observed; mussels continued to filter normally and mussel survival was 100%. Cannulas were removed from mussels after completion of the study. Mussels containing glochidia larvae in their outer gills were excluded from the study, since glochidia may affect the rate of mussel filtration (Tankersley & Dimock 1993).

Sperm Depletion Over the Course of a Mating

This experiment was conducted to estimate whether the size of ejaculates produced by male R. amarus declined over a series of consecutive ejaculations, under two treatments of sperm competition risk.

Six cannulas, three on each side, were attached to a U. tumidus mussel in the way described, with the tips of each cannula resting adjacent to the mussel’s inhalant siphon. By using six cannulas we could remove successive ejaculates without disturbing experimental fish. A single male and a female bitterling that was ready to spawn (with an extended ovipositor) were released into an experimental aquarium with an experimental mussel. The mussel was covered so that the fish could see and smell the mussel but were unable to spawn. Pairs were randomly assigned to two treatments: low sperm competition risk, where the female and territorial male were alone, or high sperm competition risk, where a single smaller rival male was added. Rival males were haphazardly selected from a pool of males and were used only once. Smaller rival males were used because male role is determined by relative size (Casalini et al. 2009), and therefore this mimicked a natural situation. Once fish had settled (territorial male courting female and displaying to rival male, female responding to courtship; typically between 30 and 60 min), the mussel was uncovered and observations started. Water samples were collected immediately after each of the first five ejaculations by the territorial male from randomly selected cannulas and the time interval between ejaculates was recorded. Collection of ejaculates did not unduly disturb spawning fish, with the interval between consecutive ejaculations by territorial males as short as 8 s.

A total of 13 replicates of each treatment were completed. Rival males were rarely able to ejaculate over the mussel, although if they did the replicate was restarted so that estimates of ejaculate size were for territorial males only. A different mussel was used for each replicate.

Sperm Depletion Over the Course of a Day

We conducted a second experiment to investigate changes in male ejaculate size in successive matings, over the course of a day of matings.

A single winged cannula was attached to a U. tumidus mussel in the way described, placed in an experimental aquarium and covered with a flowerpot. A second, exposed mussel with no cannula attached was placed together with a male bitterling and two females with extended ovipositors in the same experimental aquarium. Groups were randomly assigned to either a low (territorial male only) or high (territorial male and one rival) sperm competition risk treatment. Territorial and rival males were allowed to court the females and ejaculate over the uncannulated mussel throughout the day. Two females were used to ensure that spawnings occurred over the entire day. At three periods during the day the cannulated mussel was uncovered and the uncannulated mussel covered. We estimated territorial male ejaculate size by collecting a water sample in the way described above. After removing a water sample we again covered the cannulated test mussel and uncovered the original uncannulated mussel so that fish could continue to spawn. At the end of the experiment we counted the eggs spawned in uncannulated mussels nondestructively by gently prising the valves of the mussel apart using a mussel-opening device (Kitamura 2005).

Fish were randomly assigned to treatments, placed in aquaria, and presented with an uncannulated mussel at 0700 hours each morning that trials were conducted. The first water sample was collected in the morning (0900–1000 hours), a second at midday (1200–1300 hours) and the final sample in the afternoon (1500–1700 hours). A total of 12 replicates of each treatment were completed.

Data Analysis

All data were tested for normality using a Kolgomorov–Smirnov test and for homoscedasticity using a Bartlett test. Data that deviated from normality were ranked and the rank transformation statistic calculated (Kepner & Robinson 1988). To test for sperm depletion over the course of a mating, data were analysed using a mixed-model factorial ANOVA with repeated measures. The dependent variable was ejaculate size and we examined the effects of sperm competition risk (treatment), subject (nested within treatment), ejaculate number (repeated measure) and sperm competition risk*ejaculate number interaction. To test for sperm
depletion over the course of a day, the dependent variable was ejaculate size and we examined the effects of sperm competition risk (treatment), subject (nested within treatment), time of day (repeated measure) and sperm competition risk*time of day interaction. The numbers of eggs laid in uncanulled mussels between treatments were compared using a t test.

As a test of repeatability of sperm counts, we estimated the standard deviation of differences between repeated measurements for each count and the standard deviation of the differences between means (Bland & Altman 1999). If 95% of differences between replicates lie within 1.96 standard deviations of this estimate then differences between measurements can be considered interchangeable. In the present study all differences in counts lay within this margin, suggesting high repeatability.

RESULTS

Ejaculation Rates Under Natural Conditions

A total of 37 spawnings were observed over a 15-day period. The $X \pm SE$ rate of spawning was $4.8 \pm 0.47$ matings/h, with a $X \pm SE$ rate of ejaculation of $4.4 \pm 0.47$ ejaculations/mating. A $X \pm SE$ of $1.6 \pm 0.32$ sneaking males participated in each spawning (range 0–7), with 76% of spawnings involving at least one ejaculation by a sneaker male. There was no evidence for temporal variation in spawning or ejaculation rates during the course of the day of observations. There was no difference between observations made in the morning (0900–1300 hours; mean: 16.6 ± 1.70 min; afternoon: 18.3 ± 2.59 min; unpaired t test: $t_{35} = 0.10, P = 0.925$). There was also no difference in the mean rate of ejaculation of territorial males between morning and afternoon observation sessions ($X \pm SE$ frequency: morning: 4.3 ± 0.73 ejaculations; afternoon: 4.4 ± 0.60 ejaculations; unpaired $t$ test: $t_{35} = 0.14, P = 0.891$).

Sperm Depletion Over the Course of a Mating

There was a significant difference in the size of ejaculates over five consecutive ejaculations (Table 1), with the first ejaculation always larger than the last (Fig. 1). There was a significant difference between low and high sperm competition risk treatments (Table 1, Fig. 1); mean ejaculate size was greater when territorial males were exposed to a rival (Fig. 2). There was no significant difference between treatments (Table 2), although there was a significant interaction between treatment and time of day (Table 2); the decline in ejaculate size over a day was significantly greater when territorial males were exposed to a rival (Fig. 2). There was no significant difference in the number of eggs laid in uncanulled mussels between sperm competition risk treatments (unpaired $t$ test: $t_{22} = 1.26, P = 0.221$).

DISCUSSION

The costs of spermatogenesis are not trivial and when males mate with a succession of females they face a possibility of sperm depletion. A consequence for females of male sperm depletion is that they may receive insufficient sperm during mating to fertilize all their eggs (Wedell et al. 2002). In this study we investigated the consequences for male European bitterling fish of successive ejaculations during a mating, and successive matings over the course of a day, at two levels of sperm competition risk. Over five consecutive ejaculations the number of spermatozoa in ejaculates declined.

Table 1

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<th>Source</th>
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<th>P</th>
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<td>Subject</td>
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<td>1.07</td>
<td>0.387</td>
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<td>Ejaculate number</td>
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<td>16.81</td>
<td>&lt;0.001</td>
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<td>Sperm competition risk*ejaculate number</td>
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<td>1.13</td>
<td>0.348</td>
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Table 2

<table>
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<tbody>
<tr>
<td>Sperm competition risk</td>
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<td>0.01</td>
<td>0.925</td>
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<tr>
<td>Subject</td>
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<tr>
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<td>33.95</td>
<td>&lt;0.001</td>
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<td>2</td>
<td>6.16</td>
<td>0.004</td>
</tr>
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</table>
significantly. There was a significant effect of sperm competition risk, with mean ejaculate size significantly higher when males were exposed to a potential rival. This effect could not be explained by the length of interval between successive ejaculations, nor was there a significant effect of male size on ejaculate size. In a second experiment, ejaculate size declined over the course of a day of continuous spawning and a significant interaction between the timing of ejaculations and sperm competition risk was detected; males exposed to rivals initially produced the largest ejaculates, but over the course of a day of matings these males experienced a significantly greater decline in ejaculate size than males that were not at risk to sperm competition. This effect could not be explained by a greater number of spawnings by females exposed to two males rather than just one.

While it is often assumed that sufficient viable sperm are always released during mating, sperm depletion has been demonstrated to limit male fitness across a range of taxa (Møller 1991; Radwan 1997; Jones 2001; Preston et al. 2001; García-González 2004; Pattarini et al. 2006), and males preferred by females may be especially susceptible to sperm depletion (Warner et al. 1995; Jones 2001; Preston et al. 2001; Damiens & Boivin 2006). The results from the present study additionally demonstrate that males that respond to an increased risk of sperm competition by elevating ejaculate size and/or increasing ejaculation rate also face an increased risk of sperm depletion; allocation of sperm to current mating opportunities is traded off against ejaculate size in future matings. These results correspond with previous studies that have demonstrated sperm production to be sensitive to the risk of sperm competition, for example in the fruit fly Drosophila bifurca (Björk et al. 2007) and the marine flatworm Macrostomum sp. (Schäfer et al. 2005), although a direct link between the impact of sperm competition risk on sperm depletion has hitherto not been demonstrated empirically. A consequence of the trade-off between current and future ejaculate expenditure is seen in strategic ejaculate expenditure (Wedell et al. 2002; Pizzari et al. 2003) and sperm competition avoidance behaviour by males (Alonzo & Warner 1999; Smith et al. 2003), in combination with an increased frequency of compromised female fertility (Warner et al. 1995; García-González 2004).

The results of the present study suggest that male European bitterling are able to increase the size of ejaculates when confronted by a rival male, at least in the short term. Behavioural studies have previously demonstrated an increase in ejaculation rate in response to elevated sperm competition risk (Candolin & Reynolds 2002; Smith et al. 2002, 2003) but not ejaculate size. Notably, male R. amarus show adaptations of the testis, including a chambered sperm duct like that seen in blue-head wrasse (Shapiro et al. 1994; Rasotto & Shapiro 1998), which further suggests they may be able to control ejaculate size (Pateman-Jones 2008).

From field observations of natural spawning behaviour the risk of sperm competition for male bitterling appeared high under natural conditions, with the majority of matings involving more than one male. These field data yield an estimate of the rate of sperm releases by territorial male bitterling of 21 ejaculations/h, equating to over 200 ejaculations during 10 h of mating activity. These results suggest that experimental ejaculation rates were within the natural range for male bitterling, indicating that males face a significant risk of sperm depletion under natural conditions. There was no evidence for a temporal pattern of mating from the field study: spawning and ejaculation rates were equivalent during morning observations compared with later in the day. Consequently, changes in ejaculate size over the course of the day do not simply reflect a natural temporal pattern of declining spawning frequency, but more probably a physiological constraint on spermatogenesis.

Our findings have consequences for the mating tactics of male and female bitterling. A territorial male under natural conditions faces wide variation in the number of rivals at a mating: from solitary mating with a female, to competition with a single rival, to occasions when groups of 60 or more males may attempt to participate in a mating (Smith et al. 2000, 2002, 2003, 2004). If territorial males tailor ejaculate size to the circumstances of each spawning in which they participate, they may be able to achieve a higher reproductive success than with a fixed ejaculate size. On occasion, for example in the absence of rivals, this may entail a male conserving sperm at a cost of failing to fertilize an entire clutch of eggs (Smith & Reichard 2005). This pattern of sperm economy has been described in other species (Alonzo & Warner 2000; Petersen et al. 2001; Wedell et al. 2002; Pizzari et al. 2003). For females, attempts by males to conserve sperm that result in some of their eggs going unfertilized may prove a substantial fitness cost (Jennions & Petrie 2000; Wedell et al. 2002) and, consequently, the interests of territorial males and females may not coincide.

The response of female bitterling to the risk of reduced fertility through male sperm depletion/economy appears to be one of soliciting matings by multiple males (Smith & Reichard 2005). To do this they may have evolved a distinctive behaviour, termed skimming, which entails the female making contact with the mussel siphon with the base of her ovipositor, but without inserting the ovipositor into the mussel gill or releasing eggs. Skimming behaviour is distinct from missed ovipositions, in which the female attempts to spawn but misses the mussel siphon and deposits her eggs on the substrate (Smith et al. 2004). Skimming mimics spawning behaviour, at least to a human observer, and is thought to perform the function of signalling the willingness of a female to spawn (Smith & Reichard 2005), and serves as a cue for males to ejaculate over a mussel (Smith et al. 2007). Skimming behaviour is predicted to enhance female fitness by increasing the amount of sperm in the gill chamber of a mussel and thereby ensuring fertilization, and widening the range of

![Figure 2](#)
males that participate in mating which will tend to increase the probability that mating partners are genetically compatible (Casalini et al. 2009).

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References


