JOURNAL OF Evolutionary Biology

doi: 10.1111/jeb.12432

The function of multiple ejaculations in bitterling

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Keywords:

Acheilognathinae;
alternative mating tactics;
fertilization;
mating system;
sneaking;
sperm competition;
territoriality.

Abstract

In some taxa, males perform multiple ejaculations, which may function in sperm competition or in maintaining a baseline density of spermatozoa in the female reproductive tract to ensure fertilization, a process that has been termed 'topping up'. We investigated the function of multiple ejaculations in two species of bitterling, the European bitterling (*Rhodeus amarus*) and Chinese rose bitterling (*Rhodeus ocellatus*). Bitterling oviposit in living freshwater mussels, with fertilization taking place within the mussel gill cavity. Thus, although fertilization is external, the mussel is analogous to the female reproductive tract in an internally fertilizing species. We measured the frequency of ejaculations and mussel inspections by individual males of two bitterling species in 28 replicated mesocosms and examined focal male responses to rival ejaculations and the presence of females in spawning condition. We used a model of ejaculatory behaviour to simulate the temporal abundance of spermatozoa in mussels. Male *R. amarus* exhibited high rates of ejaculation and inspection of the siphons of mussels and increased their ejaculation rate in response to the presence of females in spawning condition. *Rhodeus ocellatus* showed lower overall rates of ejaculation, but significantly elevated ejaculation rate in response to rival ejaculations. The ejaculatory strategy of *R. amarus* is one that maintains a minimum level of spermatozoa in mussels, which is elevated when the probability of oviposition increases. In contrast, *R. ocellatus* engages more directly in sperm competition with rivals. We discuss these results in the context of the function of multiple ejaculations and male mating tactics.

Introduction

Sperm competition, competition between the sperm of two or more males for the fertilization of ova, is an important mechanism of sexual selection that has shaped the evolution of mating systems (Parker, 1990). Sperm competition is a form of post-copulatory male–male competition that occurs in both internal and external fertilizers (Pitnick & Hosken, 2010) and imposes selection on male behaviour, as well as sperm and seminal fluid characteristics (Pitnick *et al.*, 2009; Pizzari & Parker, 2009). Empirical and theoretical work in recent years has demonstrated the significance of sperm competition for sexual selection and mating system evolution (Birkhead & Møller, 1998; Parker, 1998; Parker & Pizzari, 2010).

Sperm competition theory seeks to predict optimal sperm allocation strategies for males in relation to the risk and intensity of sperm competition. The *risk* of sperm competition is the probability that a male's sperm will compete with the sperm of other males. The *intensity* of sperm competition is the extent of overlap of the ejaculates of different males and therefore is a function of the number of males that engage in sperm competition and the quantity of sperm they contribute to a mating (Parker, 1998; Wedell *et al.*, 2002).

The sperm allocation tactics of males are relatively well understood (Pizzari & Parker, 2009). Thus, males are predicted to allocate sperm differentially according to the circumstances of each mating in which they participate, that is, the risk and intensity of sperm competition and the quality of the female (Wedell *et al.*,

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2002; Pizzari *et al.*, 2003). It also pays males to invest more in females with higher fecundity (Reinhold *et al.*, 2002). Given that the fitness gain from mating with any particular female decreases with increasing investment, it additionally pays for males to invest in novel females, a phenomenon known as the 'Coolidge effect' (Dewsbury, 1981; Wedell *et al.*, 2002). Sperm allocation strategies are also mediated by male social status. Dominant and subordinate males differ in their ability to attract and monopolize females and experience different levels of sperm competition risk (Leach & Montgomerie, 2000; Cornwallis & Birkhead, 2006; Rudolfsen *et al.*, 2006; Parker & Pizzari, 2010).

In some taxa, mating can involve multiple ejaculations from one or several males. The significance of multiple ejaculations, rather than a single large release of sperm as a mating tactic, was considered by Lanier *et al.* (1979) and Parker (1984, 1998). Parker (1984) proposed two functions for multiple ejaculations. In species with internal fertilization, and in which spermatozoa experience passive loss from the female reproductive tract, multiple ejaculations may be more effective in sperm competition than a single large ejaculate. This will particularly be the case if the loss or death of spermatozoa in the female reproductive tract is rapid (Parker, 1984). The other functional explanation for multiple ejaculations is that males may 'top-up' or replenish their sperm in the reproductive tract of a female if spermatozoa undergo passive loss and if there is a critical quantity of sperm required to fertilize a female's eggs. Multiple intermittent ejaculations have the effect of maintaining the quantity of spermatozoa above the threshold for fertilization. In this case, multiple ejaculations are predicted even in the absence of sperm competition (Parker, 1998).

These explanations for the function of multiple ejaculations are not mutually exclusive, and both are contingent on the temporal loss of viable spermatozoa from the reproductive tract of the female. The adaptive function of multiple ejaculations raises intriguing questions in the context of male ejaculatory tactics, but has received limited attention and has yet to be explored empirically.

We investigated the ejaculatory tactics of two species of bitterling fish, the European bitterling (*Rhodeus amarus*) and Chinese rose bitterling (*Rhodeus ocellatus*). Bitterling are freshwater fish that spawn and incubate their eggs in the gills of living freshwater mussels, with fertilization taking place within the mussel gill cavity. Bitterling display high rates of ejaculatory behaviour, with multiple ejaculations associated with a single spawning event. Whereas fertilization is external in bitterling, in the context of experimental studies of reproduction, the mussel can be considered analogous to the female reproductive tract in an internally fertilizing species (Spence *et al.*, 2013), but with the advantage that it is under experimental control, making bitterling usually amenable for studies of sperm competition and fertilization dynamics.

The mating systems of *R. amarus* and *R. ocellatus* are ostensibly similar, although that of *R. amarus* is better understood. For further details on the reproductive biology of *R. amarus*, see Smith *et al.* (2004). The two species are considered polygynandrous, with both males and females making multiple mate choice decisions with multiple partners in several different mussels. Dominant males aggressively defend territories to monopolize mussels and lead females to a defended mussel for spawning (Wiepkema, 1961; Smith *et al.*, 2004). Females use long ovipositors to place their eggs into the gills of a mussel through the mussel's exhalant siphon. Females inspect mussels before spawning, basing their spawning site choices on mussel and male quality (Casalini *et al.*, 2009; Agbali *et al.*, 2010, 2012), with consistent preferences for certain mussel characteristics that relate to enhanced embryo survival (Smith *et al.*, 2000, 2001; Spence & Smith, 2013). Males fertilize the eggs by ejaculating over the inhalant siphon of the mussel. Water filtered by the mussel carries the spermatozoa to the eggs where they are fertilized and complete development in 3–4 weeks (Spence & Smith, 2013). Pre-oviposition ejaculation, whereby males ejaculate into the siphon of a mussel before a female spawns, is a common feature of the mating system. The spermatozoa of *R. amarus* and *R. ocellatus* remain viable within a mussel gill for a prolonged period, being rich in mucins (Pateman-Jones *et al.*, 2011), and in *R. amarus* at least, are known to be capable of fertilizing eggs at least 14 min after ejaculation (Reichard *et al.*, 2004a). The risk of sperm competition in bitterling is high, with the majority of spawnings under natural conditions involving more than one male (Smith & Reichard, 2005; Reichard *et al.*, 2009; Smith *et al.*, 2009). Those males that control access to mussels enjoy high reproductive success, although this success is eroded at high male densities (Reichard *et al.*, 2004a,b, 2005, 2009). Male dominance is determined by body size (Smith *et al.*, 2003; Reichard *et al.*, 2008; Casalini *et al.*, 2009, 2013), with smaller males adopting alternative mating tactics, although these roles are not fixed and male mating behaviour is opportunistic (Smith *et al.*, 2002; Řežucha *et al.*, 2012). Males respond to the resulting sperm competition in accordance with theoretical predictions (Parker *et al.*, 1996), elevating their ejaculation rate when competing with a single rival, but reducing their ejaculate expenditure with increasing numbers of competing males (Smith *et al.*, 2003, 2009, 2013). Dominant males also invest more ejaculations in novel mussels, in accordance with predictions for a Coolidge effect, the mussel representing a new fertilization opportunity (Casalini *et al.*, 2013; Spence *et al.*, 2013).

We investigated the ejaculatory behaviour of male *R. amarus* and *R. ocellatus* in 28 replicated mesocosms and measured their responses to rival ejaculations, the

presence of females in spawning condition and the mussel species available for oviposition. Fecundity, egg and body size are equivalent, and male and female mating behaviours are qualitatively indistinguishable (Pateman-Jones *et al.*, 2011). The two species are also phylogenetically closely related (Okazaki *et al.*, 2001; Kawamura *et al.*, 2014). Despite this, male *R. amarus* display a substantially higher rate of ejaculation than *R. ocellatus*, which has been linked to their shorter breeding season (Pateman-Jones *et al.*, 2011).

The aim of the study was to investigate the function of multiple ejaculations in these two species of bitterling. In the case that multiple ejaculations function primarily in sperm competition, we predicted that males would respond to rival ejaculations by elevating their ejaculatory expenditure. In contrast, if multiple ejaculations play a primary role in 'topping-up' *sensu* Parker (1984), we predicted multiple ejaculations in the absence of sperm competition, though with ejaculatory behaviour sensitive to the probability of oviposition by females.

Materials and methods

Study system

Rhodeus amarus used in the study were collected from the River Kyjovka in the south-east of the Czech Republic. *Rhodeus ocellatus* were captive bred from a stock of fish imported from the River Yangtze Basin, China.

Freshwater mussels used in the study were *Anodonta anatina*, *A. woodiana* and *Unio tumidus*, which were collected by hand at the start of the bitterling spawning season from the River Kyjovka. *Anodonta anatina* and *U. tumidus* are native European mussel species. *Anodonta woodiana* is an Asian species of mussel that has recently been introduced to Europe (Watters, 1997), but which overlaps in its natural distribution with *R. ocellatus* (Jing & Zimin, 2013). Both *R. amarus* and *R. ocellatus* can use all three mussel species for oviposition.

Mesocosms

A total of 28 experimental mesocosms were established in large fibreglass pools (1.3 × 1.3 m) located in the garden of the Institute of Vertebrate Biology, Czech Republic. Each pool was filled to a depth of 600 mm with tap water and furnished with a gravel substrate, artificial plants as refuges and four sand-filled plastic pots. Each pot contained a mussel; pots kept mussels in a fixed position while permitting them to adopt a natural orientation. The mesocosms were stocked with 4 male and 4 female bitterling. Half the mesocosms ($n = 14$) were stocked with *R. amarus* and the remaining 14 with *R. ocellatus*. Pairs of mussels of each species were assigned randomly to mesocosms, with 14 receiving a combination of two *A. anatina* and two *U. tumidus*, whereas the remaining 14 received one *A. anatina*, one *U. tumidus* and two *A. woodiana*. In this way, half the mesocosms contained two species of native European mussel (*A. anatina* and *U. tumidus*) or two native and two Asian mussels (*A. woodiana*), with the same combinations of mussel species presented to each bitterling species. The experimental design is summarized in Table 1.

Fish were fed daily with a mixture of frozen chironomid larvae and copepods. Fish and mussels in mesocosms were exposed to natural light and temperature variation, typical for mid-May in central Europe. Mean (± SD) temperature was 19.3 (± 1.8) °C, and day length was approximately 15.5 h.

Behavioural observations

Every mesocosm was observed on two separate occasions, each for 15 min, with a mean (± SD) interval of 2.5 (± 1.1) days between successive observations. During each observation, the behaviour of a randomly selected territorial male was scored. During behaviour recording distinguishing features of the focal male, such as fin damage, discoloured or dislodged scales, or the presence of parasites, were recorded to permit previously observed males to be avoided during the second period of observation. A record was kept of the exact timing of ejaculations by the focal male over the guarded mussel and also of the frequency of ejaculations by other males over the same mussel. The frequency of inspection of the guarded mussel by the focal male, whereby the fish orientates itself with its snout close to the exhalant siphon, apparently sampling water leaving the mussel (Smith *et al.*, 2004), was also scored. A record was made of the presence of any females with extended ovipositors in the mesocosm, which indicates

Table 1 Experimental design of the study, showing bitterling and mussel species combinations, total number of mesocosms and number of fish recorded in 15-min behavioural observation sessions. *Rhodeus amarus*, *Anodonta anatina* and *Unio tumidus* all overlap naturally in their European distribution, whereas *R. ocellatus* and *Anodonta woodiana* overlap in Asia. Both *R. amarus* and *R. ocellatus* can oviposit in all three mussel species.

Bitterling species	Mussel species	Number of mesocosms	Number of fish recorded
R. amarus	2 × *A. anatina* 2 × *U. tumidus*	7	14
R. ocellatus	2 × *A. anatina* 2 × *U. tumidus*	7	14
R. amarus	1 × *A. anatina* 1 × *U. tumidus* 2 × *A. woodiana*	7	14
R. ocellatus	1 × *A. anatina* 1 × *U. tumidus* 2 × *A. woodiana*	7	14

a readiness to spawn, during the period of observation. Females remain in spawning condition for approximately 24 h, or until they have spawned their entire clutch of ovulated eggs, which they do so over the course of 5–10 ovipositions. During the time they display an extended ovipositor, they move actively among male territories, inspecting mussels and males.

A total of 56 replicates were completed, each on a different male guarding a different mussel. After completion of an observation, the total shell length (TL) of the guarded mussel was measured to the nearest 1 mm. To avoid undue disturbance to the fish in the mesocosm, focal male standard length (SL) was judged by eye to the nearest 1 mm. Whereas these estimates may not have been accurate on an absolute scale, they permitted the relative size of the males within mesocosms to be distinguished. No oviposition events were observed during behaviour recording.

Statistical analysis

Mesocosm observation data were analysed using a Generalized Linear Mixed Model (GLMM) with a Poisson distribution applied to the count of ejaculations as the response variable. Alternative models were compared using the Akaike information criterion (AIC). All analyses were conducted using the *lme4* library in R (Bates *et al.*, 2014; R Core Development Team, 2014). From an initial data exploration (Zuur *et al.*, 2010), no evidence was found for influential outlying observations, collinearity between explanatory variables and nonlinear relationships between explanatory variables and the response variable. Because two observations were taken from each mesocosm a mixed model with mesocosm as a random term was applied, this approach accounted for the lack of independence between observations within mesocosms.

A main model was selected *a priori*, based on the results of previous research, which predicted that competing males and the presence of females in spawning condition would influence focal male ejaculatory behaviour for both bitterling species. The goal of the analysis was to examine whether responses varied between bitterling species and whether factors interacted. The main model took the form:

$$Ejac_{ij} \sim Poisson(\mu_{ij})$$

$$E(Ejac_{ij}) = \mu_{ij} \text{ and } var(Ejac_{ij}) = \mu_{ij}$$

$$\log(\mu_{ij}) = \eta_{ij}$$

$$\eta_{ij} = \alpha + \beta \times Insp_{ij} + \beta \times Bitt_{ij} + \beta \times Ovi_{ij} + \beta \times Rejac_{ij} + \beta \times Bitt : Ovi_{ij} + \beta \times Bitt : Rejac_{ij} + \beta \times Rejac : Ovi_{ij} + a_i$$

$$a_i \sim N(0, \sigma^2)$$

Each observed ejaculation by a focal male (*Ejac*) (subscript *j*) was indexed per mesocosm (subscript *i*). The main model contained a linear effect for mussel inspection by the focal male (*Insp*) as a main term and interactions for the factors: bitterling species (*Bitt*), presence or absence of female with an extended ovipositor (*Ovi*) and rival male ejaculation over the mussel guarded by the focal male (*Rejac*). The model term a_i is a random term for each mesocosm. Working from the main model, 16 derived models of varying complexity were compared (Table 2). For the best-fitting model,

Table 2 Model comparisons showing model number, model description, degrees of freedom (d.f.), ΔAIC and model weight (*w*).

Model ID	Model description	d.f.	ΔAIC	*w*
M1	*Insp* + *Bitt* + *Ovi* + *Rejac* + *Bitt* × *Ovi* + *Bitt* × *Rejac* + *Ovi* × *Rejac*	9	0.00	0.766
M2	*Insp* + *Bitt* + *Ovi* + *Rejac* + *Insp* × *Bitt* × *Ovi* + *Insp* × *Bitt* × *Rejac*	13	2.55	0.214
M3	*Insp* + *Bitt* + *Rejac* + *Bitt* × *Rejac*	6	7.48	0.018
M4	*Insp* + *Bitt* + *Ovi* + *Rejac*	6	13.72	0.001
M5	*Insp* + *Ovi* + *Rejac* + *Ovi* × *Rejac*	6	14.31	0.001
M6	*Insp* + *Rejac* + *Insp* × *Rejac*	5	20.43	0.000
M7	*Insp* + *Bitt* + *Ovi* + *Bitt* × *Ovi*	6	20.94	0.000
M8	*Insp* + *Ovi* + *Insp* × *Ovi*	5	23.21	0.000
M9	*Ovi* + *Rejac* + *Ovi* × *Rejac*	5	25.33	0.000
M10	*Bitt* + *Rejac* + *Bitt* × *Rejac*	5	26.22	0.000
M11	*Insp*	3	28.71	0.000
M12	*Insp* + *Bitt* + *Insp* × *Bitt*	5	30.29	0.000
M13	*Bitt* + *Rejac* + *Bitt* × *Rejac*	5	31.21	0.000
M14	*Rejac*	3	38.28	0.000
M15	*Ovi*	3	41.22	0.000
M16	*Bitt*	3	51.34	0.000

AIC, Akaike information criterion.

residuals were examined to ensure model assumptions were met and that effects were adequately accounted for by the model.

Inclusion of the covariates focal male size and mussel size, and the factor mussel species made no contribution to the fit of any models and are not considered further in the analysis.

Ejaculation model predictions

We used the bitterling ejaculation model of Smith & Reichard (2013) to simulate the temporal pattern of spermatozoa abundance in mussels for *R. amarus* and *R. ocellatus*. The model predicts the number of spermatozoa in the gill cavity of a mussel following ejaculation by a male, estimated from an empirically derived spermatozoa–time relationship. A Holling type IV rational function was fitted to these data, which took the form:

$$A_{(t)} = \frac{\alpha t^2}{\beta + \gamma t + t^2}$$

where $A_{(t)}$ is the abundance of sperm in the mantle cavity at time t in seconds. Fitted parameters were $\alpha = 12672$, $\beta = 40.8$, $\gamma = 0.0031$. The model assumed that sperm competition is a 'fair raffle' *sensu* Parker (1990); that is, each male's fertilization probability is equivalent to the number of his spermatozoa in the mussel mantle cavity following oviposition. For full model details, see Smith & Reichard (2013).

Model predictions were based on the mean timing of ejaculations by focal males from the mesocosm study in response to the factors *Ovi* and *Rejac*. The model was formulated specifically for *R. amarus*, although spermatozoa characteristics of this species appear, superficially at least, to be comparable to the related *R. ocellatus* (Pateman-Jones, 2008; Pateman-Jones *et al.*, 2011).

Results

Mesocosm study

A comparison between models showed that those that included mussel inspection by the focal male (*Insp*), rival ejaculations (*Rejac*) and the interaction between bitterling species and rival ejaculations (*Bitt* × *Rejac*) (M1, M2, M3 in Table 2) performed better than all other models (ΔAIC 0.00, 2.55 and 7.48, respectively). This outcome implies that these covariates were key variables in explaining the ejaculation frequency of focal males. Thus, focal male ejaculatory behaviour was significantly positively associated with the presence of a female with an extended ovipositor (*Ovi*) and with the focal male's own mussel inspection behaviour (*Insp*), irrespective of bitterling species (Table 2). In the case of *R. ocellatus*, there was also a positive association between ejaculation by a rival (*Bitt* × *Rejac*) on focal male ejaculation rate (Table 2).

Most AIC weights were associated with the main model (M1), which included the continuous variable *Insp* and the main terms and interactions for the factors *Rejac*, *Bitt* and *Ovi*. This model was also assessed as the best single model based on a ΔAIC threshold of < 2. The second best model, ΔAIC = 2.55 (M15), included all main terms and two 3-way interactions (*Insp* × *Bitt* × *Ovi* and *Insp* × *Bitt* × *Rejac*). The ΔAIC value of 2.55 implies that despite its additional complexity, this model provided a poorer fit to the data than the main model (M1). The main model (M1) showed *Ovi* to be a key explanatory variable (Table 3; Fig. 1a), in addition to *Insp* (Table 3; Fig. 2), and the interaction between *Bitt* and *Rejac* (Table 3; Fig. 1b).

Ejaculation model

The ejaculation model of Smith & Reichard (2013) predicted comparable patterns of spermatozoa abundance in mussels for the two bitterling species in response to *Ovi* and *Rejac*, though with some noteworthy differences. In *R. amarus*, the mean interval between ejaculations in mesocosms was shorter than for *R. ocellatus* (Fig. 3a,b), with males maintaining a density of spermatozoa in defended mussels at a density that rarely fell below 10 000 spermatozoa (Fig. 4a). Once a rival ejaculated in a guarded mussel, the observed interval between ejaculations declined (Fig. 3b), with the predicted baseline of spermatozoa in the mussel rising to a minimum exceeding 20 000 spermatozoa (Fig. 4a). In contrast, male *R. ocellatus* were predicted to maintain spermatozoa at densities < 5000 spermatozoa in the absence of rival spermatozoa. However, when a rival succeeded in ejaculating in the guarded mussel, the minimum baseline increased to a comparable level to that of *R. amarus* at > 20 000 spermatozoa (Fig. 4b).

The response of the two bitterling species to a female in spawning condition also diverged. In the absence of a female with an extended ovipositor, the observed interval between ejaculations was comparable for both

Table 3 Estimates and statistical significance of fixed effects from model M1 (*Insp* + *Bitt* + *Ovi* + *Rejac* + *Bitt:Ovi* + *Bitt* × *Rejac* + *Ovi* × *Rejac*). All other tested models exceed a ΔAIC threshold of 2.

Model parameter	Estimate	SE	*z*	*P*
Intercept	−1.727	0.627	−2.756	0.006
Insp	0.045	0.013	3.504	<0.001
BittRO	−1.007	0.897	−1.123	0.261
Ovi1	1.755	0.644	2.725	0.006
Rejac1	0.585	0.846	0.691	0.489
BittRO × *Ovi1*	−0.918	0.819	−1.120	0.263
BittRO × *Rejac1*	2.126	0.700	3.038	0.002
Ovi1 × *Rejac1*	−0.556	0.864	−0.644	0.520

AIC, Akaike information criterion.

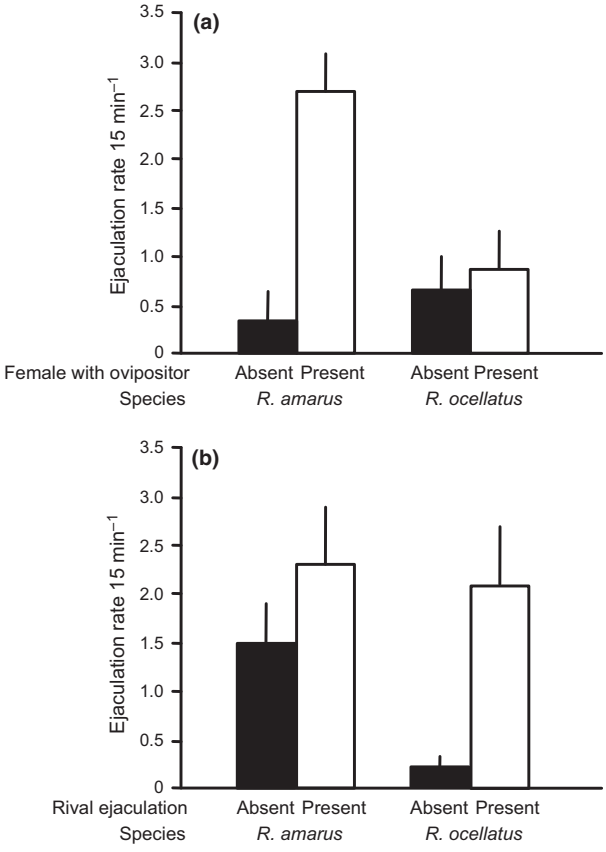

Fig. 1 Mean (+ 1 SE) ejaculation rate (15 min^{-1}) of territorial male *Rhodeus amarus* and *R. ocellatus* over a guarded mussel. (a) With and without a female with an extended ovipositor present. (b) With and without ejaculates of rival males over the same mussel.

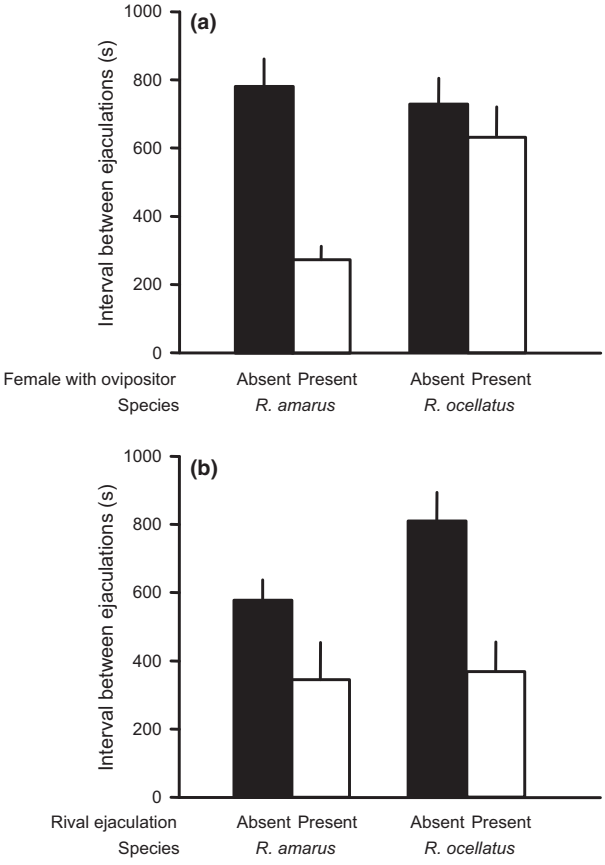

Fig. 3 Mean (+ 1 SE) interval (s) between ejaculations by territorial male *Rhodeus amarus* and *R. ocellatus* over a guarded mussel. (a) With and without a female with an extended ovipositor present. (b) With and without ejaculates of rival males over the same mussel.

Fig. 2 Mean (+ 1 SE) inspection rate (15 min^{-1}) of the exhalant siphon of a guarded mussel by territorial male *Rhodeus amarus* and *R. ocellatus*.

bitterling species, with both predicted to maintain a minimal sperm density in mussels (at ~5000 spermatozoa) (Fig. 5a). However, in the presence of a female with an extended ovipositor, *R. amarus* increased their mean rate of ejaculation to a greater extent than *R. ocellatus* (Fig. 3a). The consequence was that the predicted density of spermatozoa in mussels was markedly elevated in the case of *R. amarus* (Fig. 5a), but to a lesser degree in *R. ocellatus* (Fig. 5b).

Discussion

We examined the role of multiple ejaculations in the ejaculatory tactics of two species of bitterling housed in mesocosms at densities that reflected those in nature. Multiple ejaculations during mating are not uncommon in vertebrates, particularly in mammals (Eaton, 1978; Dewsbury, 1984; Parker, 1984). The function of multiple mating has typically been associated with sperm competition, with an assumption that ejaculate size

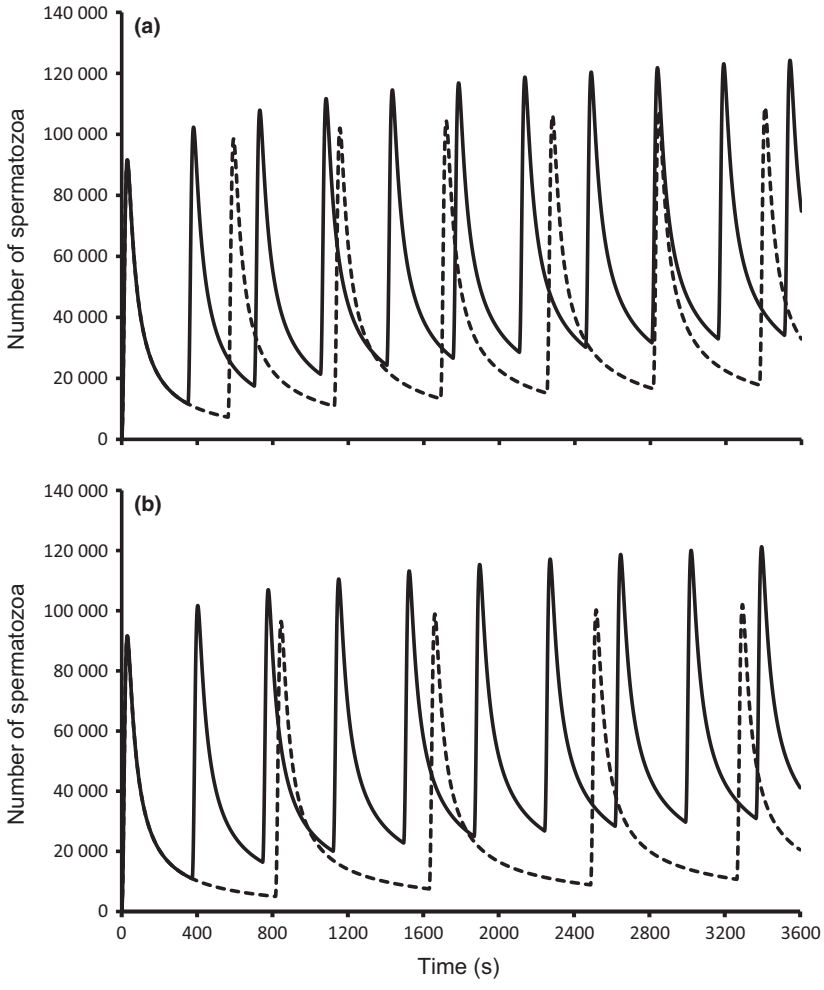

Fig. 4 Predicted number of territorial male spermatozoa in the gill cavity of a guarded mussel with (solid line) and without (dashed line) the ejaculates of rival males over the same mussel. (a) *Rhodeus amarus*. (b) *R. ocellatus*.

cannot be readily manipulated by the male so that the most efficient way to deliver an elevated volume of sperm to the female reproductive tract is through repeated mating. Parker (1984) suggested an alternative explanation for multiple ejaculations, that of a male maintaining a baseline abundance of spermatozoa in the female reproductive tract to ensure fertilization when ovulation occurs. Parker's (1984) model assumed that the frequency of ejaculations correlated with the rate at which the viability of spermatozoa declined, with males 'topping up' their sperm in the female reproductive tract at a rate that correlated with the longevity ('fertilizing life') of their spermatozoa. There have hitherto been no empirical tests of 'topping up' in animal mating systems.

Multiple ejaculations were a feature of the mating tactics of both bitterling species in the present study, with evidence of both 'topping-up' mussels with spermatozoa *sensu* Parker (1984), as well as a role in sperm competition. Thus, males of both species maintained a minimum level of spermatozoa in the gill cavities of mussels they guarded in the way predicted for 'topping up' to operate. In both species, simulations of spermatozoa numbers in mussels based on the model of Smith & Reichard (2013) predicted that with the mean rate of ejaculation observed in mesocosms, spermatozoa number did not fall to zero, even in the absence of sperm competition. Both species responded to an elevated intensity of sperm competition when rival males ejaculated in guarded mussels by increasing their ejaculation frequency.

We also detected species differences in ejaculatory tactics. In the absence of sperm competition, *R. amarus* maintained a greater density of spermatozoa in mussels than *R. ocellatus*, by 'topping-up' mussels with sperm more frequently. *Rhodeus amarus* were also more responsive to the presence of spawning females and 'topped up' mussels more frequently than *R. ocellatus*. However, *R. ocellatus* did respond to the intensity of sperm competition, raising spermatozoa density in mussels to a comparable level to *R. amarus* in response to rival ejaculation. Thus, our broad conclusions are that

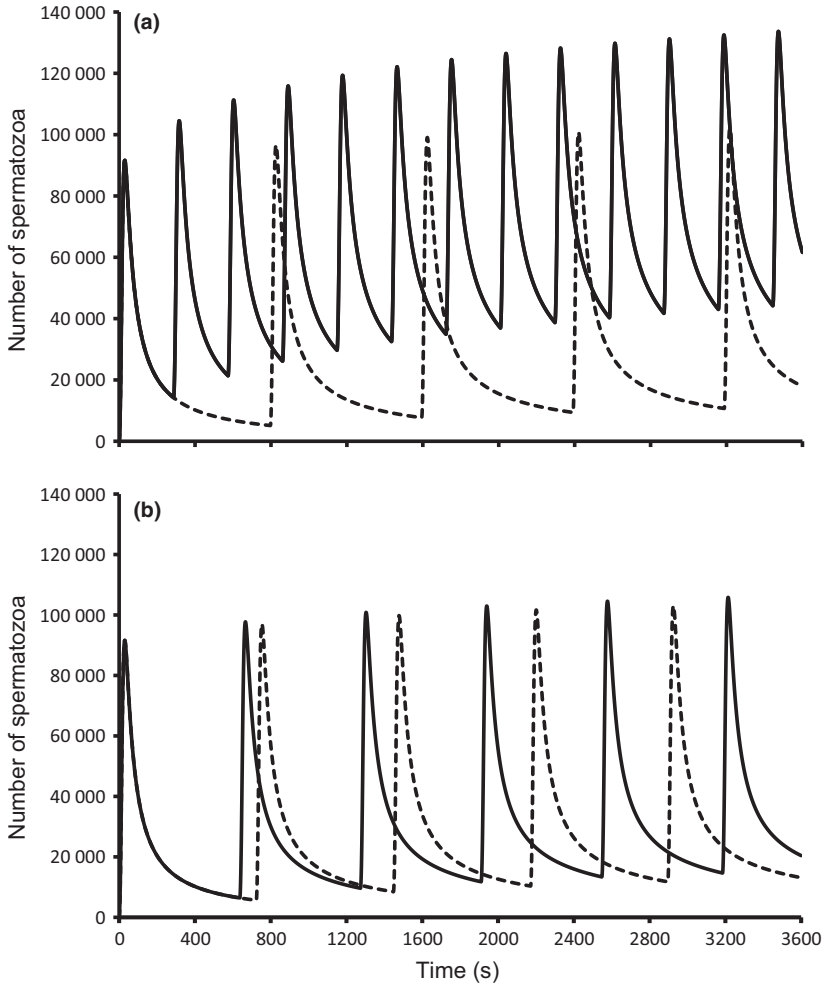

Fig. 5 Predicted number of territorial male spermatozoa in the gill cavity of a guarded mussel with (solid line) and without (dashed line) a female with an extended ovipositor present. (a) *Rhodeus amarus*. (b) *R. ocellatus*.

multiple ejaculations, in both *R. amarus* and *R. ocellatus*, play a role in 'topping-up' mussels with spermatozoa, but also function in response to sperm competition.

The function of 'topping-up' mussels with spermatozoa appears to be to maintain a baseline density of viable spermatozoa in the gills of a mussel that will ensure sperm precedence should a female oviposit in the mussel guarded by a male. Pre-oviposition ejaculations appear to play a significant role in the reproductive success of male bitterling. In a study of parentage in a seminatural population of *R. amarus*, Reichard *et al.* (2004a) demonstrated that every embryo assigned parentage could potentially have been fathered solely through pre-oviposition ejaculations. 'Topping up' also enables a male to fertilize eggs even if he is not present at oviposition. As such, it represents an efficient alternative male mating tactic, enabling a male to compete with rivals, but without investing in courtship or territoriality. The contrary is also the case, and 'topping up' is also an effective response to the risk of 'sneaky' mating attempts by rivals. Bitterling sperm persists in the gills of freshwater mussels because it contains abundant mucins (Pateman-Jones *et al.*, 2011). The function of mucins is to slowly release motile spermatozoa over an extended period after ejaculation through the gradual dissolution of the mucin in water (Marconato *et al.*, 1996; Scaggiante *et al.*, 1999; Mazzoldi, 2001). By extending the longevity of spermatozoa, mucins greatly increase the period over which fertilization can occur. The location of bitterling sperm in mussels is unclear, but it may adhere to the mantle tissue or gills (Casalini *et al.*, 2009; Agbali *et al.*, 2010). The evolution of mucilaginous sperm may be an adaptation to prevent the rapid removal of spermatozoa from the mussel through filtration. However, this feature of bitterling sperm also facilitates pre-oviposition ejaculation and 'topping up' as a male mating tactic.

Male *R. amarus* ejaculated at a high rate and proved sensitive to the presence of females that were ready to spawn. Sperm release over mussels was regular, even in the absence of sperm competition. In contrast, *R. ocellatus* had a lower overall ejaculation rate but

responded strongly to the ejaculatory behaviour of rivals. In the absence of sperm competition, male *R. ocellatus* released sperm into mussels less frequently. Thus, these two bitterling species showed comparable ejaculatory tactics, though with minor, but notable differences.

In *R. amarus*, multiple ejaculations appeared to function primarily in 'topping-up' spermatozoa in the mussel gill in accordance with the predictions of Parker (1984). This species was conspicuously more responsive to the presence of a female with an extended ovipositor and overall had a higher ejaculation rate than *R. ocellatus*. In contrast, *R. ocellatus* was most responsive to sperm competition from rivals and elevated its ejaculation rate primarily in response to rival ejaculations. These observations correspond with the findings of Pateman-Jones *et al.* (2011), who showed that in comparison with *R. ocellatus* and the related *Rhodeus sinensis*, *R. amarus* displayed the most developed reproductive apparatus and a number of traits associated with both high levels of sperm production and high fertilization efficiency, implying that this species has a greater capacity to engage in multiple ejaculations than *R. ocellatus*. The major differences in the mating systems of *R. ocellatus* and *R. amarus* are in the length of spawning season. The spawning season for *R. amarus* lasts approximately 6 weeks (Smith *et al.*, 2004), in contrast to 6 months for *R. ocellatus* (Kanoh, 2000; Kitamura, 2005). The mean spawning season fecundities for both species are similar, at approximately 150–250 eggs depending on female size (Nagata, 1985; Smith *et al.*, 2004; Kitamura, 2005), and clutch sizes are comparable, with a mean ± SE clutch size per spawning for *R. amarus* of 2.9 ± 1.2 eggs (Smith *et al.*, 2000) and for *R. ocellatus* 4.4 ± 1.3 eggs (Reichard *et al.*, 2007). The shorter spawning season of *R. amarus* means that spawnings are temporally clustered over a short, but relatively intense breeding season, resulting in a higher frequency of matings per unit time than the more protracted breeding season of *R. ocellatus*. An outcome is that male *R. amarus* will have shorter intervals between matings than *R. ocellatus* and face a greater risk of sperm depletion. In this situation, the favoured ejaculatory tactic in *R. amarus* appears to be one of maintaining a relatively high baseline of spermatozoa in mussels and to respond with further ejaculations when the probability of a female ovipositing, cued by the presence of a female with an extended ovipositor, is increased. Because of the more protracted breeding season, maintaining a high baseline level of spermatozoa in mussels may be too energetically costly for *R. ocellatus*. The strategy in this species appears to be one of maintaining a lower level of 'topping up' and responding primarily to sperm competition from rivals by elevating ejaculation rate.

The system by which male bitterling, of both *R. amarus* and *R. ocellatus*, are able to maintain spermatozoa numbers in mussels at a species-specific baseline density is not yet clear. Males may possess an endogenous mechanism to measure the passage of time between ejaculations. Alternatively, they may use olfactory cues to measure sperm density in mussels. A striking feature of spawning behaviour of both male and female bitterling is the frequency with which they position themselves at the exhalant siphons of mussels, termed 'inspection' behaviour (Smith *et al.*, 2004). In the case of females, this is explicable in terms of the female assessing mussel quality as a site for oviposition, particularly as a female is likely to be unfamiliar with a given mussel. In contrast, males should be familiar with mussels they guard. Despite this, they examine the siphons routinely, even in the absence of a female, which discounts the role of this behaviour in courtship, although the function of male inspection of mussels has hitherto been unclear. In the present study, male *R. amarus* inspected the exhalant siphons of guarded mussels with a mean ± SE interval of 90 ± 14 s. In the case of *R. ocellatus*, the interval was 120 ± 17 s. In both species, this equates to approximately five inspections per ejaculation. At this frequency, the only obvious functional change in mussel condition is the density of spermatozoa in its gills. Thus, we tentatively propose that the function of mussel inspection by males is to assess their spermatozoa density, an hypothesis that lends itself to experimental investigation.

An assumption in using the sperm competition model of Smith & Reichard (2013) was that male ejaculatory behaviour corresponded with ejaculation, although this may not always have been the case. Some males may have been infertile, or failed to release sperm for some other reason. A result would be that they would not attain sperm representation in the mussel gill cavity. Previous studies in other taxa have demonstrated that matings without sperm release can be a feature of mating systems (e.g. Løvlie *et al.*, 2005). An additional possibility is that male bitterling may perform 'deceptive' ejaculations to encourage female oviposition, without investing in an ejaculate (Pateman-Jones, 2008). Deceptive ejaculatory behaviour may be adaptive to males if they risk sperm depletion, and the behaviour enables them to conserve sperm and thereby participate in further matings. However, the behaviour loses its adaptive value in the face of sperm competition, and a prediction would be that males would not engage in deceptive ejaculatory behaviour when a male rival was present at a spawning, which was typically the case in the present study.

Despite designing this study to test for an effect of mussel species on male ejaculatory behaviour, we failed to detect one. European *R. amarus* appeared not to differentiate in their response to European or Asian mussels, whereas Asian *R. ocellatus* also failed showed a differential response. Female bitterling do show more marked reactions to different species of mussel and are

generally more discriminatory than males in making oviposition decisions (Reichard *et al.*, 2007, 2010, 2012; Casalini *et al.*, 2013). Further research will explore the impact of species differences in mussel filtration rates and their implications for spermatozoa longevity in the mussel gill cavity.

In conclusion, we demonstrated that multiple ejaculations have a dual function in bitterling. Multiple ejaculations permit a male to maintain a baseline of spermatozoa in mussels, the site of oviposition, in accordance with the predictions of Parker (1984) for 'topping up'. Multiple ejaculations also function in escalating investment in a given mating by increasing the number of spermatozoa in a mussel gill cavity as a response to an increase in the probability of oviposition occurring and the intensity of sperm competition. Male inspection of mussels may permit assessment of sperm density in mussels based on olfactory cues. Whereas 'topping up' has not been recognized as a feature of animal mating systems, it may be an overlooked aspect of the ejaculatory tactics of both internal and external fertilizers.

Acknowledgments

We are grateful to Rowena Spence and Andre Philips for comments and Jacek Radwan and two anonymous referees for constructive reviewing. Research was supported by CSF Grant 13-05872S.

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Data deposited at Dryad: doi:10.5061/dryad.k2661

Received 4 March 2014; revised 4 May 2014; accepted 9 May 2014