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A sperm competition model for the European bitterling (*Rhodeus amarus*)

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Abstract
Sperm competition occurs when the spermatozoa of one male coincide with those of another to fertilise the same eggs. 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 fertilisation, a process that has been termed 'topping up'. We investigated multiple ejaculations in the European bitterling (*Rhodeus amarus*), a freshwater fish that oviposits in freshwater mussels. We quantified spermatozoa in the mussel mantle cavity following ejaculation, and measured sperm motility parameters of males adopting different mating tactics. Following ejaculation spermatozoa density in the mussel increased linearly, peaked after 30 s, and then declined exponentially. Spermatozoa motility parameters did not differ between male mating tactics. We parameterised a model of sperm competition for *R. amarus*, which accurately predicted male fertilisation probability. We discuss these results in the context of multiple ejaculations and male mating tactics.

Keywords
Acheilognathinae, alternative mating tactics, mating system, sneaking, sperm competition, sperm motility.

1. Introduction

Sperm competition, competition between the sperm of two or more males for the fertilisation of ova, is an important mechanism of sexual selection that has shaped the evolution of mating systems (Parker, 1990; Andersson, 1994). Sperm competition is a form of postcopulatory male–male competition that occurs in both internal and external fertilisers (Pitnick & Hosken, 2010), and

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imposes selection on 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 & 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 first considered by Parker (1984; reviewed 1998). There are two possible functions of this behaviour. The first is that in species with internal fertilisation, 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 spermatozoa loss 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 undergoes passive loss and if there is a critical quantity of sperm required to fertilise a female's eggs. Multiple intermittent ejaculations have the effect of maintaining the quantity of spermatozoa above the threshold for fertilisation. In this case multiple ejaculations are predicted even in the absence of sperm competition (Parker, 1998).

Both explanations for multiple ejaculations are not mutually exclusive, and both are contingent on the temporal loss of viable spermatozoa from the reproductive tract of the female. Baker & Bellis (1993) proposed that topping up may account for multiple ejaculations in human reproductive behaviour. However, we are not aware of any experimental studies that have investigated topping up as a mating tactic in animals.

Here we parameterise and test a model of multiple ejaculations in the European bitterling (*Rhodeus amarus*), a small freshwater fish that spawns and incubates its eggs in the gills of freshwater mussels. In *R. amarus*, and other bitterling species, fertilisation takes place within the mussel gill cavity. Thus, while fertilisation is external, in the context of experimental studies of reproduction the mussel can be considered as an extension of the female reproductive tract in an internally-fertilising species (Spence et al., 2013). The bitterling mating system is promiscuous; both males and females spawn repeatedly with multiple partners and in multiple mussels. Dominant males aggressively defend territories to monopolise 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), with consistent preferences for certain mussel characteristics that relate to enhanced embryo survival (Smith et al., 2000, 2001; Spence & Smith, 2013). Males fertilise the eggs by repeatedly ejaculating over the inhalant siphon of the mussel. In a field study the rate of ejaculation by territorial male *R. amarus* was estimated to be 21 ejaculations h^{-1} throughout the day during the breeding season, with a mean $\pm$ SE rate of 4.4 ± 0.47 ejaculations at each mating attempt with a single female (Smith et al., 2009). Water filtered by the mussel carries the sperm to the eggs where they are fertilised and complete development in 3–4 weeks (Aldridge, 1999). Pre-oviposition ejaculation, whereby male *R. amarus* ejaculate into the siphon of a mussel before a female spawns, is a common feature of male courtship and mating. Bitterling spermatozoa remain viable within a mussel gill for a prolonged period, being rich in mucins (Pateman-Jones et al., 2011), and are capable of fertilising eggs up to 14 min after ejaculation (Reichard et al., 2004a). The risk of sperm competition is high, with the majority of spawnings under natural conditions involving more than one male (Smith et al., 2009). Those males that control access to mussels enjoy high reproductive success, though this success is eroded at high male densities (Reichard et al., 2004a, b, 2005). Male dominance is determined by body size (Smith et al., 2003; Casalini et al., 2009), with smaller males adopting alternative mating tactics, although these roles are not fixed and male mating behaviour is opportunistic (Candolin & Reynolds, 2002; 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 (Candolin & Reynolds, 2002; Smith et al., 2003, 2004, 2009). Dominant males also invest more ejaculations in novel mussels, in accordance with predictions for a Coolidge effect, the mussel representing a new fertilisation opportunity (Spence et al., 2013). For further details on the reproductive biology of *R. amarus* see Smith et al. (2004).

To provide further insights into sperm competition in bitterling, and particularly the potential adaptive role of multiple ejaculations, we conducted two studies. The first described the temporal pattern of spermatozoa number

in the mussel mantle cavity following ejaculation, the second tested whether sperm motility parameters differed between males adopting contrasting mating tactics (guarder vs. non-guarder). Using the results of these studies we parameterised a sperm competition model to predict male fertilisation probability, and validated the model against behavioural and parentage data from a mesocosm study.

2. Material and methods

2.1. Study system

All *R. amarus* used in the study were collected from the River Kyjovka in the southeast of the Czech Republic. Fish were transported in river water to the Institute of Vertebrate Biology in Brno and held in a garden pond. Freshwater mussels used in the study were *Unio tumidus*, which were collected at the start of the bitterling spawning season by hand from an oxbow lake close to the River Kyjovka, a tributary of the River Dyje. The mussels were transported to Brno and held in a second garden pond that did not contain bitterling, approximately one month before the start of the study. *U. tumidus* are widespread and common in central Europe and frequently used by *R. amarus* for spawning (Smith et al., 2000). Experimental work was conducted in the aquarium facility at the Institute of Vertebrate Biology. During experiments, which were conducted in May and early June, fish were exposed to a natural diurnal cycle of approximately 15:9 h light/dark and temperature range of 17–24°C (Smith & Reichard, 2005).

2.2. Sperm depletion in mussels

A study was conducted to estimate the change in sperm density over time within the mantle cavity of a mussel following ejaculation over the inhalant siphon by a male bitterling. The study was intended to quantify spermatozoa depletion over time to enable estimates of sperm competition intensity to be made.

Using a sharp file a 2-mm-long, 1-mm-wide hole was carefully made in the shell of a *U. tumidus* mussel. A BD Venflon winged cannula (Becton Dickinson Infusion Therapy, Helsingborg, Sweden) was inserted into the opening and the needle removed from the cannula sheath. The tip of the cannula was gently positioned so that it rested inside the mantle cavity 5 mm from the inhalant siphon. The cannula wings were glued to the mussel shell

to hold it in position. A 1 m long piece of 5 mm diameter silicon tubing was fixed to each cannula and a syringe fitted to the open end. We observed no detrimental effects to mussels of fitting cannulas, which continued to filter normally and mussel survival was 100%. No mussels containing glochidia larvae in their outer gills were used, as glochidia may affect mussel filtration (Tankersley & Dimock, 1993). After completion of experiments the cannulas were removed.

To make estimates of spermatozoa density in the mussel mantle cavity a single male *R. amarus* was released into an experimental aquarium measuring 60 × 40 × 40 cm (depth) with an uncannulated *U. tumidus* to elicit territorial behaviour. A female with an extended ovipositor was gently released in the experimental aquarium and a mussel with a cannula fitted replaced the original mussel. Males were allowed to ejaculate once over the inhalant siphon of the experimental mussel before a single water sample was collected from the mantle cavity using the cannula after a pre-determined time interval. Intervals were: 0, 10, 30, 60, 120, 240 and 600 s following ejaculation. Following ejaculation, and after the appropriate time interval, 300 mm^3 (0.3 ml) of water was collected from the mantle cavity 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) and a count made of the number of spermatozoa in the sample using a binocular microscope (Nikon Eclipse E200) with a 40× objective. Counts were made of sperm cells in five 1 × 1 × 0.1 mm squares to obtain a mean spermatozoa density for the subsample and multiplied up to the entire sample volume of 300 mm^3 to give an estimate of total spermatozoa number. A volume of 300 mm^3 of water was sufficient to collect the entire ejaculate released into the mussel, since collecting a larger volume did not yield higher estimates of total ejaculate size (Smith et al., 2009). For each sample, three samples were counted to yield a mean estimate of spermatozoa number. Estimates of ejaculate size were made for six males at every time interval using a different mussel on each occasion. Only a single water sample was collected from an ejaculate so that estimates of spermatozoa density at different time intervals were derived for different ejaculates. A minimum of one hour was allowed to elapse between ejaculations to ensure estimates of spermatozoa density were not affected by previous ejaculations into a mussel. The order in which water

samples for different time intervals following ejaculation were collected was randomised. A record was made of fish body length (measured from the tip of the snout to the origin of the tail fin) and mussel shell total length. Females did not spawn during trials and spermatozoa counts were all based solely on pre-oviposition ejaculations.

2.3. Sperm motility

To measure whether males that adopted different mating tactics produced spermatozoa that varied in motility we used computer-assisted analysis of spermatozoa. A group of *R. amarus* were collected from the River Kyjovka and held in a garden pond at the Institute of Vertebrate Biology in Brno where they were provided with mussels and allowed to spawn. After two weeks, a sample of 26 male *R. amarus* were separated into two size classes by eye and randomly paired in individual aquaria measuring 75 $\times$ 40 $\times$ 40 cm (depth). Aquaria had a sand substrate, contained artificial plants and were aerated continuously. Each pair was provided with a single *U. tumidus* mussel and in each case the larger male established territorial dominance and defended the mussel from its smaller rival. A female in spawning condition was added to each aquarium and the fish were allowed to spawn over a two-day period, with the larger male consistently playing the territorial guarder role during spawnings, while the smaller male attempted sneak fertilisations in a non-guarder role (Smith et al., 2004, 2009). Under natural conditions males switch roles regularly and European bitterling do not display morphological differences or distinct syndromes of behaviour associated with either mating role (Řežucha et al., 2012).

To measure sperm quality, males were killed by cutting the spine at the base of the skull and one testis removed, placed in 20 μl of water and disrupted, using a pair of mounted needles, to release the spermatozoa. The remaining testis was left within the body cavity. 1 μl of the sperm/water mixture was immediately (within 15 s) added to a 12-well multitest slide (ICN, Basingstoke, UK) for tracking. Spermatozoa were videotaped for 4 min using an Olympus BH-2 microscope with a $\times$40 objective (negative-high phase contrast) linked to a Sony Hyper HAD black and white video camera with a charge-coupled diode iris via an MTV-3 adaptor. After completion of videoing the procedure was repeated using the second testis to provide two sets of data for each male. All samples were analyzed at room temperature.

Taking sperm samples directly from the testis has been used routinely in comparable studies with fish (e.g., Gage et al., 2005; Janhunen et al., 2009), and appears to provide a reliable assessment of the sperm available to a male for mating. Spermatozoa released in ejaculates is extremely variable among ejaculations in bitterling (Smith et al., 2009; Pateman-Jones et al., 2011), with the quality of sperm released potentially affected by sperm depletion, female quality, female behaviour, and the presence of male rivals, thereby making ejaculated spermatozoa an unreliable measure of overall spermatozoa quality in males (Wedell et al., 2002).

Video footage was analysed using CASA (version 7V2B) in conjunction with the Hobson Sperm Tracker (Hobson Vision, Baslow, UK). CASA settings were optimised for bitterling spermatozoa, and spermatozoa from each sample were tracked for 22 $\times$ 15 s intervals. The following spermatozoa motility variables were examined: VCL, curvilinear velocity (μm s^{-1}), the sum of incremental distances moved by a spermatozoa in each frame divided by total track time; VSL, straight line velocity (μm s^{-1}), the straight line distance between the start and end points of the track, divided by track time; LIN, linearity (%), the straight line distance between the start and end points of the track, divided by the sum of the incremental distances along the actual path; MOT, motility (%), the number of motile spermatozoa divided by the total number of spermatozoa in the analysis field. These motility parameters were selected because they have been demonstrated to correlate with fertilisation efficiency in fish (Hala et al., 2009). Motility variables for each testis were averaged to provide a single estimate of each variable for each male.

2.4. Data analysis

The correlation between male body length and peak number of spermatozoa in the mussel mantle cavity was tested using Pearson's correlation, with residuals tested for normality using a Shapiro–Wilk test. Paired *t*-tests were used to compare spermatozoa quality parameters between mating tactics. Pearson's correlation was used to test the correlation between male body length and spermatozoa motility parameters. In this case a single male was inadvertently not measured and consequently these correlations were based on 23 degrees of freedom rather than 24. Pearson's correlation was also used to test the relationship between observed and model-predicted male reproductive success using square-root transformed data.

3. Results

3.1. Spermatozoa depletion in mussels

Immediately following ejaculation the number of spermatozoa in the mantle cavity of a mussel increased rapidly, peaking after approximately 30 s. Thereafter the number of spermatozoa declined exponentially. After 10 min spermatozoa were still detectable in samples, but at an abundance over an order of magnitude lower than at the peak (Figure 1). There was no significant correlation between male body length or mussel total length and peak number of spermatozoa in the mussel (Pearson's correlation, male body length $r_5 = 0.57$, $p = 0.232$; mussel total length $r_5 = 0.01$, $p = 0.987$). Mean ± SE male body length was 48 ± 2.4 mm and mussel total length 103 ± 2.4 mm.

3.2. Spermatozoa motility

We detected no significant difference between guarding and non-guarding males in spermatozoa motility parameters; VCL (paired t-test, $t_{12} = 1.65$, $p = 0.125$), VSL ($t_{12} = 0.83$, $p = 0.424$), LIN ($t_{12} = 1.25$, $p = 0.234$), MOT ($t_{12} = 0.79$, $p = 0.445$) (Figure 2). Male body length did not correlate

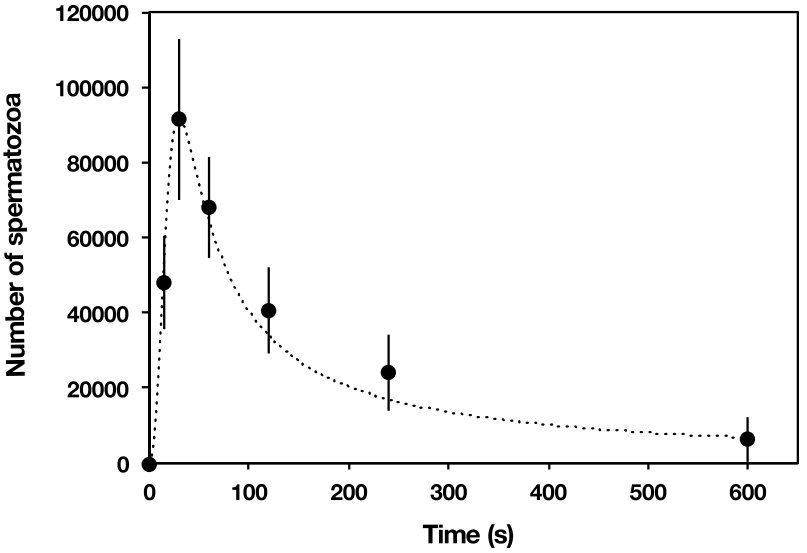

Figure 1. Mean (± 95% CI) number of spermatozoa of *R. amarus* in the mantle cavity of a freshwater mussel after different time intervals following ejaculation. The dotted line is the number of spermatozoa based on model predictions.

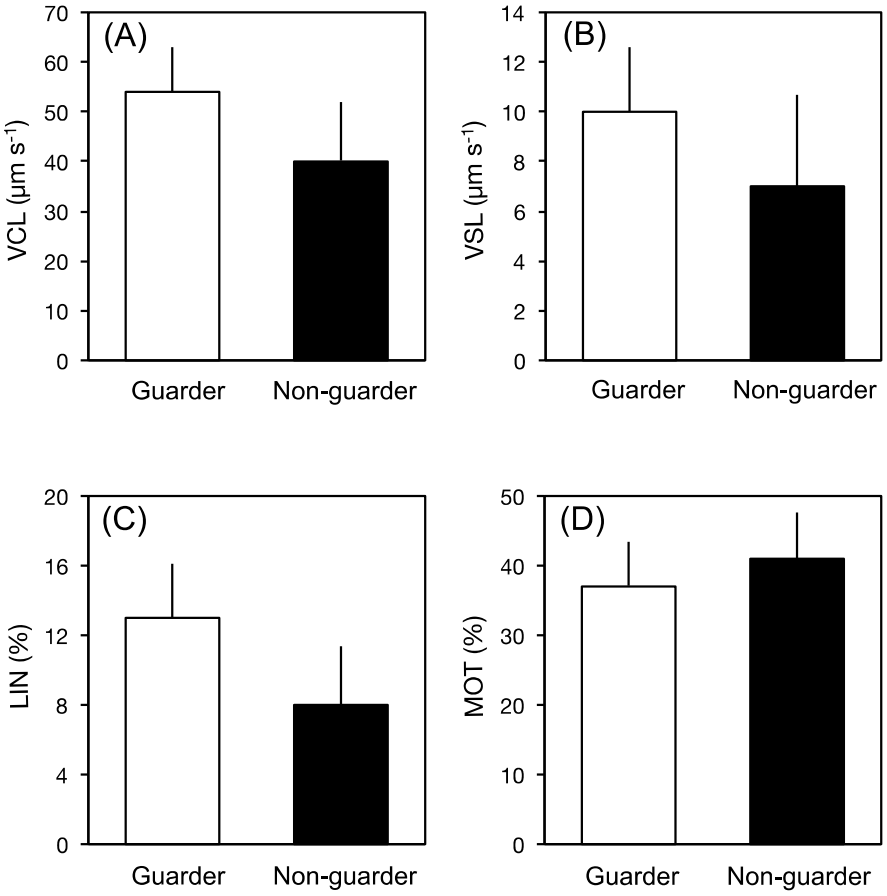

Figure 2. Mean (± 1 SE) spermatozoa motility variables of guarder and non-guarder male *R. amarus*: (A) VCL: curvilinear velocity (μm s^{-1}); (B) VSL: straight-line velocity (μm s^{-1}); (C) LIN: linearity (%); (D) MOT: motility (%).

with any motility parameters (VCL, Pearson's correlation ($r_{23} = 0.14$, $p = 0.492$); VSL ($r_{23} = 0.11$, $p = 0.615$); LIN ($r_{23} = 0.20$, $p = 0.344$); MOT ($r_{23} = -0.12$, $p = 0.583$)).

3.3. Sperm competition model

The number of spermatozoa in the gill cavity of a mussel following ejaculation by an individual male was estimated from the sperm depletion study using the empirically derived spermatozoa-time relationship (Figure 1). We fitted a Holling Type IV rational function to these data (Turchin, 2003),

a reaction-diffusion model, 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. Parameters were obtained by non-linear least-squares estimation in Stata v11.1 for Mac to be $\alpha = 12\,672$, $\beta = 40.8$, $\gamma = 0.0031$ ($r^2 = 0.70$) (Figure 1). This function is a spatial ecological model that is used to model predator-prey interactions, as well as population growth-decline and reaction-diffusion effects. The model was selected because it provided a good representation of the data and is easy to compute (Bolker, 2008). In addition, like other rational functions, the model reaches a finite asymptote, which in the context of the current study would be when all spermatozoa are removed from the mussel.

If the assumption is made that sperm competition is a 'fair raffle' *sensu* Parker (1990); i.e. that each male's fertilisation probability is equivalent to the number of his spermatozoa in the mussel mantle cavity immediately following oviposition, then this function permits prediction of male probability of fertilisation success given the timing of ejaculations and oviposition.

3.4. Model analysis

A sensitivity analysis was conducted in which the model parameters were altered $\pm 10\%$ of parameter estimates. The parameter sensitivities were compared using a sensitivity index:

$$S = \left(\frac{\left(\frac{R_\mathrm{a} - R_\mathrm{n}}{R_\mathrm{n}}\right)}{\left(\frac{P_\mathrm{a} - P_\mathrm{n}}{P_\mathrm{a}}\right)} \right),$$

where R_a and R_n are the model responses to the altered and nominal parameters, and P_a and P_n are the altered and nominal parameters respectively (Haefner, 1996). The results show a modest tendency for nonlinear responses to the parameter changes (Table 1). Sensitivity to changes in parameter values were either approximately equivalent (in the case of α), or less (in the case of β and γ) than the proportional change in parameter estimates, indicating that model predictions were robust over a broad range of parameter values (Haefner, 1996).

The density of a guarder male's spermatozoa in the mantle cavity of a mussel relative to that of a rival was dependent on the relative timing

Table 1.
Sensitivity of sperm competition model to model parameters.

Parameter	Sensitivity	
	+	−
α	1.10	0.93
β	0.35	0.30
γ	0.69	0.65

For calculation see text. The sensitivity values correspond to a 10% increase (+) and decrease (−) in parameter estimates.

of ejaculation. When the interval between ejaculations was short, relative spermatozoa density was predicted to be comparable for both males. If the interval was longer, the mantle cavity contained only the spermatozoa of the guarder male until ejaculation by the non-guarder, after which the number of non-guarder spermatozoa exceeded that of the guarder, with the magnitude a function of the delay between ejaculations (Figure 3). This pattern of spermatozoa density in relation to timing of ejaculation was reflected by predicted fertilisation probability. Guarder probability of fertilising a clutch of eggs was predicted to be comparable in the case that guarder and rival ejaculated into a mussel within a short time of each other, irrespective of timing of oviposition (Figure 4A). However, with a longer interval between ejaculations the relative time at which oviposition occurs is more critical. Thus, a male whose rival ejaculates 10 s after them will achieve a probability of fertilising a clutch of eggs a little below 0.5 no matter when oviposition occurs (Figure 4A). However, if a rival ejaculates after 180 s, the guarder will experience a fertilisation probability as low as <0.2 if oviposition occurs after 200 s. For the rival male, fertilisation probability is simply the converse of that of the guarder male (Figure 4B). In a non-guarder role, a long delay before ejaculating after the guarder means that fertilisation probability will be zero if oviposition occurs during that interval, but is greater if oviposition occurs promptly after they ejaculate, and especially if the interval between ejaculations is an extended one. Thus, the model predicts that to achieve the highest probability of fertilising a batch of eggs both guarder and non-guarder males should maximise the interval between their own and

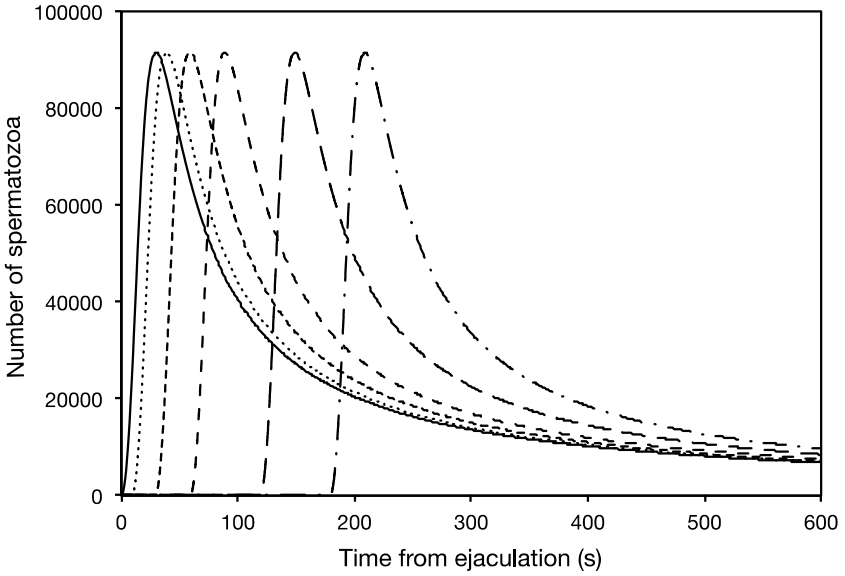

Figure 3. Predicted number of spermatozoa of *R. amarus* in the mantle cavity of a freshwater mussel at different time intervals. Guarder male (solid line) ejaculation at time 0. Rival male ejaculation at 10 s (dotted line), 30 s (short-dashed line), 60 s (medium-dashed line), 120 s (long-dashed line) and 180 s (dashed and dotted line) following ejaculation by the guarder male.

their rival's ejaculations, while ensuring that their own ejaculations coincide with, or ideally immediately precede, oviposition.

3.5. Model validation

Using the sperm competition model we made predictions of male reproductive success for a series of experimental matings involving multiple males in which paternity analysis was conducted. The design of the study is reported in Reichard et al. (2004b) and the paternity analysis in Reichard et al. (2004a). The same results are not repeated here. The study was conducted in a 45 000-l outdoor pool stocked with a population of >200 *R. amarus* caught from the River Kyjovka. During the study a diver recorded fish behaviour from a distance of approximately 1.5 m. exact timings of ejaculations by territorial males and individually distinguished rivals were recorded. Once a spawning occurred, the precise time was noted and a cylindrical (diameter 80 cm) net set around each spawning arena was gently raised. All the fish retained in the net were captured using a hand net, which always included

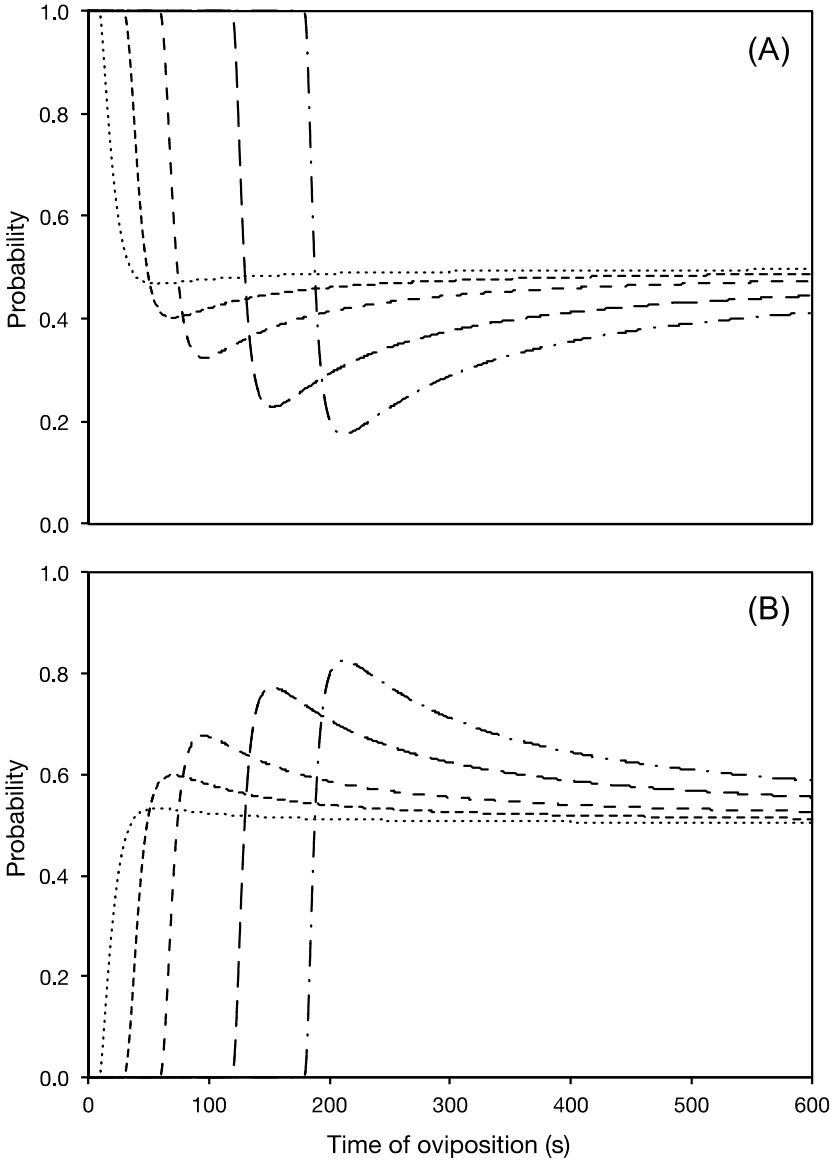

Figure 4. Predicted fertilisation probability for (A) a guarder male in competition with a rival that ejaculates 10 s (dotted line), 30 s (short-dashed line), 60 s (medium-dashed line), 120 s (long-dashed line) and 180 s (dashed and dotted line) after guarder male ejaculation; (B) a non-guarder male that ejaculates 10 s (dotted line), 30 s (short-dashed line), 60 s (medium-dashed line), 120 s (long-dashed line) and 180 s (dashed and dotted line) after ejaculation at time 0 by a guarder male.

the territorial male, female and some, though not always all, non-guarding males. The mussel in which spawning occurred was isolated and after 7 days embryos were removed and fixed in 96% ethanol. Parentage of embryos was assigned using standard protocols (Reichard et al., 2004a). A total of 10 spawnings involving more than a single male were recorded and parentage assigned among participants. Using behavioural data from this study we used our sperm competition model to predict male fertilisation probability on the basis of the timing of oviposition and ejaculations by all participating males. We assumed that sperm competition was a fair raffle and that each male's contribution of sperm was directly proportional to the number of eggs fertilised. Because fertilisation is not instantaneous, we estimated the proportion of eggs fertilised by each male to be dependent on the quantity of each male's spermatozoa present in the mussel mantle cavity for 30 s immediately following oviposition. Predictions were compared with observed guarder male fertilisation success estimated by parentage analysis by Reichard et al. (2004a). Both the precision (Pearson's correlation on square-root transformed data, $r_9 = 0.64$, $p = 0.046$) and accuracy of the model (slope = 0.74) revealed a close match between observed and predicted reproductive success (Figure 5). A plot of the residuals against fitted values showed no

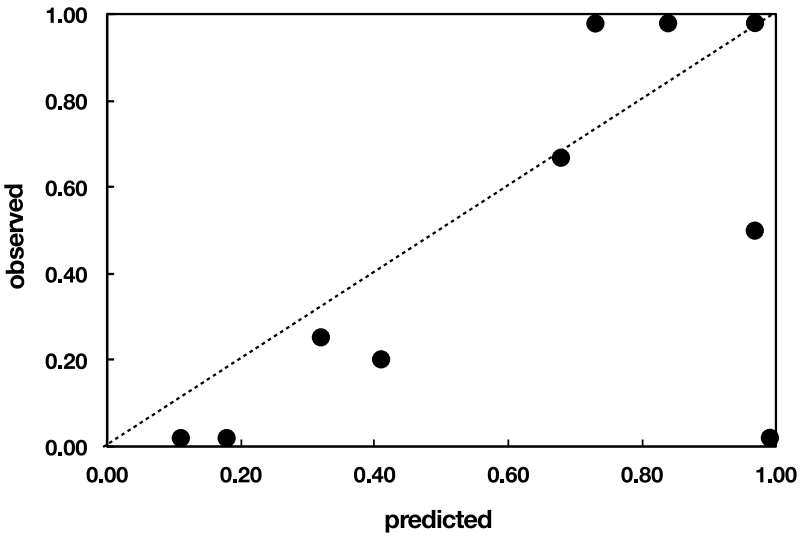

Figure 5. Observed guarder fertilisation success based on parentage analysis versus predicted fertilisation probability based on model predictions. Each point represents a different spawning event. The 1:1 line is indicated with a dotted line.

trend and no significant deviation from normality (Shapiro–Wilk normality test: $W = 0.93$, $p = 0.274$).

4. Discussion

The first aim of this study was to describe the temporal pattern of spermatozoa number in the mantle cavity of a mussel following ejaculation by male *R. amarus*. The change in spermatozoa number showed a distinctive and predictable pattern. Immediately following ejaculation the number of spermatozoa increased rapidly, peaked after 30 s, and subsequently declined exponentially (Figure 1). Male bitterling ejaculate over the inhalant siphon of the mussel so that the sperm is drawn into the siphon with the inhalant flow. The delay from ejaculation to maximum spermatozoa density in the mussel mantle cavity is presumably a function of the distance at which ejaculation occurs, as well as the time taken for the surrounding water to pass down the inhalant siphon. Spermatozoa were recovered in quantifiable numbers within 15 s of ejaculation (Figure 1), though some were presumably inside the mantle cavity and capable of fertilising eggs before that point.

Ejaculates released by a male are sometimes momentarily visible and can be seen to be drawn into the mussel siphon. Bitterling sperm is denser than water and males always ejaculate over the top of the inhalant siphon so that the sperm sinks on to the siphon and is entrained by the inhalant flow. There is good circumstantial evidence that sufficient spermatozoa remain in the mussel mantle cavity to fertilise eggs after at least 14 min, the period after ejaculation that males were shown by parentage analysis to have fathered embryos in natural spawnings (Reichard et al., 2004a). Indeed a striking feature of Reichard et al.'s (2004a) study was that every embryo assigned parentage could potentially have been fathered solely through pre-oviposition ejaculations.

The unusual longevity of bitterling spermatozoa in the mussel mantle cavity cannot be attributed to the inefficiency of the mussel to clear particles through filtration. A feature of *R. amarus* ejaculates is that it contains abundant mucins (Pateman-Jones et al., 2011). Mucous seminal fluid is relatively rare in fishes, but has been reported in gobies, toadfish, cottids, blennies and catfishes (Marconato et al., 1996; Mazzoldi, 2001). The function of mucins in fertilisation 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). By extending the longevity of spermatozoa, mucins greatly increase the period over which fertilisation can occur. Thus, the extended period over which spermatozoa were recovered from mussels is probably a reflection of their gradual release from mucilaginous sperm. The location of bitterling sperm in the mantle cavity of mussels is unclear, but it may adhere to the mantle tissue, or possibly accumulate on the gills (Agbali, 2011). This adaptation may have evolved to prevent the rapid removal of spermatozoa from the mussel through filtration, however it also facilitates pre-oviposition ejaculation as a male mating tactic.

The second aim of the study was to test whether sperm motility parameters differed between males adopting contrasting mating tactics. We detected no evidence for a difference in sperm motility parameters, and this finding corresponds with other results that suggest male mating tactics in *R. amarus* are conditional, with no major morphological or physiological differences between males adopting either a guarding or non-guarding role (Smith et al., 2003, 2004; Pateman-Jones et al., 2011; Řežucha et al., 2012). Some minor behavioural differences have been recorded, however. For example, Spence et al. (2013) showed that while guarder males responded to a new mussel by increasing their ejaculation rate, non-guarders did not. Řežucha et al. (2012) also recorded a weak tendency for guarder males to have greater testis mass after controlling for body size. The lack of any substantial differences among males reflects the fact that they frequently switch mating tactics, sometimes over the course of a single mating, and all but the smallest males will play the role of both guarder and non-guarder repeatedly over the course of a breeding season (Smith et al., 2004).

Using the results of these studies we parameterised a sperm competition model to predict male fertilisation probability, and validated the model against behavioural and parentage data from a mesocosm study. The model predicted paternity with surprising accuracy (with some exceptions, see below), which implies that our assumptions for sperm competition in *R. amarus* were reasonable. Thus, sperm competition in *R. amarus* appears to be a fair raffle, with each male's fertilisation probability equivalent to the number of his spermatozoa in the mussel mantle cavity in the 30 s immediately following oviposition. Another assumption was that ejaculate size was consistent among ejaculations. Experimental evidence suggests that ejaculate size declines over multiple ejaculations, and also that ejaculate size is larger if males mate in the presence of a rival (Smith et al., 2009). In our validation study

the maximum number of ejaculations by any male was eight, which is too few to detect a decline in ejaculate size (Smith et al., 2009). All matings also involved more than a single male so that, while these variables were not accommodated in the model, they were unlikely to strongly influence model predictions.

In some cases the model failed to predict parentage accurately. Thus, in two cases guarder males were predicted to achieve close to 100% fertilisation success, but achieved only 50 and 0%. This inaccuracy may partly be a function of the low batch fecundity of *R. amarus*, typically just 3 eggs, resulting in small-sample effects and thereby increasing error rate. In addition, males may occasionally release sperm too far from the mussel siphon for the entire ejaculate to be 'captured' by the mussel, though we have no data to indicate whether this does occur. However, males do sometimes disturb the mussel during sperm release by touching the shell, or passing too close to the mussel so that it detects water movements in its immediate proximity. In these cases the mussel quickly closes its siphons and briefly ceases filtration. A consequence is that the entire ejaculate may not enter the mussel, with the result that ejaculates released when the mussel is undisturbed and filtering normally will have a greater probability of fertilisation. In the present study we did not record whether males disturbed mussels in cases where model predictions deviated substantially from observed results. An additional assumption was that fertilisation was 100%. There is evidence that this is not always the case in *R. amarus*, at least when just a single male attempts to fertilise the eggs in a mating (Smith & Reichard, 2005). However, in all cases in the present study more than a single male participated in mating, and fertilisation rates can reasonably be assumed to be effectively 100%.

Multiple ejaculations by male *R. amarus* appear to function in sperm competition when multiple males participate in a spawning. However, given the depletion of spermatozoa in the mussel mantle cavity that we observed, an additional prediction is that guarder males should maintain a minimum density of spermatozoa in mussels in their territory, in accordance with Parker's (1998) concept of "topping up". A striking feature of the *R. amarus* mating system is that males systematically patrol mussels in their own territory, as well as those of their neighbours, examine the exhalant siphons of mussels and often ejaculate over them (Smith et al., 2004). This behaviour takes place even in the absence of females, though the presence of a female with

an extended ovipositor significantly increases the rate of inspection and ejaculation, as well as male aggression (Konečná et al., 2010). What information males obtain by examining mussel siphons is unclear, but it may provide them with cues about the abundance of their own, and possibly rival, spermatozoa. In addition, while oviposition is unpredictable, mussel inspection by a female with an extended ovipositor, and especially 'skimming' behaviour, whereby a female sweeps over a mussel but without inserting her ovipositor (Smith et al., 2007), are cues that might be used by males to increase sperm expenditure in a mussel. Inspection of mussels may also provide a male with information about mussel filtration rates, which vary significantly among the mussel species used by *R. amarus* for oviposition (Smith et al., 2001). Thus, different mussels may clear spermatozoa from their mantle cavities at different rates, and males would be predicted to tailor their rates of sperm replenishment in response to these differences by modulating their rates of ejaculation. A further prediction, then, is that topping up rates will be higher for *Unio pictorum*, the species with the highest rates of filtration, and lowest for *Anodonta cygnea*, the species with the lowest filtration rates. *U. tumidus* and *A. anatina* are predicted to be intermediate between these two (Smith et al., 2001).

From the perspective of female mating decisions, if females attend to male ejaculation rates they could potentially bias paternity towards a particular male. Female *R. amarus* are sensitive to the presence of different numbers of males, and perform behaviours that increase the probability that more than one male will participate in a mating (Kanoh, 2000; Smith & Reichard, 2005). Females are also choosy about which males they will mate with, though male dominance appears frequently to override female mating preferences (Reichard et al., 2005; Casalini et al., 2009; Agbali et al., 2010). However, by matching oviposition to preferred male ejaculations the females have the capacity to exert cryptic choice (Eberhard, 1996), or at least to balance the probability of fertilisation towards particular males.

In a broader context, the function of multiple ejaculations is typically associated with sperm competition. However, the evidence from the present study also implicates a role for topping-up *sensu* Parker (1984). Thus, in species in which multiple ejaculations are a feature, the challenge is to identify a role for topping up in the mating system, as distinct from sperm competition. Candidates are taxa with internal fertilisation (or, as in the case of bitterling, is analogous to internal fertilisation), in which the risk of sperm

competition may be low, where conception is unpredictable, but where sperm deposited in the female reproductive tract depletes over time.

In conclusion, we showed that spermatozoa abundance in freshwater mussels used by *R. amarus* for oviposition followed a distinctive and predictable pattern. Sperm motility parameters that correlate with fertilisation efficiency did not vary with male mating tactic. We modelled male fertilisation probability as a fair raffle and model predictions accurately predicted observed male reproductive success. The model provides support for a role of multiple ejaculations in both sperm competition and in topping up mussels with sperm in anticipation of oviposition. This study highlights the central importance of pre-oviposition ejaculation in the mating system of *R. amarus*, and our model generates testable predictions for the response of male ejaculation rate in response to risk of sperm competition, probability of oviposition, and mussel quality.

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