Spatial distribution of oviposition sites determines variance in the reproductive rate of European bitterling (*Rhodeus amarus*)

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Summary

We investigated reproductive rate in relation to oviposition site distribution and quality in the European bitterling, *Rhodeus amarus*, a freshwater fish that spawns on the gills of living unionid mussels. In a laboratory experiment male bitterling led females to groups of four mussels at a significantly higher rate than single mussels, irrespective of mussel species. Females spawned significantly more frequently on the gills of mussels in groups than on solitary mussels, and showed a preference for spawning on the gills of *Unio pictorum* in comparison with *Anodonta anatina*. In a field experiment the total number of eggs spawned on the gills of four mussels was significantly higher than that of single mussels, though the mean number of eggs *per* mussel was equivalent within species. There was a significant effect of species on the number of eggs spawned in mussels; *U. pictorum* and *U. tumidus* received more eggs than *A. anatina* and *A. cygnea*. We discuss these results in the context of mating system evolution.

Keywords: mating system, opportunity for selection, oviposition, sexual selection.

Introduction

The temporal and spatial distribution of fertilisations is a key determinant of mating system evolution (Emlen & Oring, 1977; Clutton-Brock, 1989;

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Ligon, 1999; Shuster & Wade, 2003). Males often defend patches of resources critical for female reproduction, and females tend to aggregate around those resources in space and time, with direct consequences for male mating success. Thus, the strength of sexual selection and, therefore, mating system evolution, will be dependent on the spatial and temporal distribution of fertilizations. For example, in the strawberry poison frog, *Dendrobates pumilio*, nursery sites can be limiting for female reproduction. Females gather around suitable nursery sites, while males focus their activity in areas where female density is highest. Males compete for access to sites with high female density, which determines male reproductive success (Pröhl & Berke, 2001). Similarly, in the Cape ground squirrel, *Xerus inauris*, low temporal clustering of oestrous females results in a small number of males within colonies being able to monopolize receptive females, thereby leading to high variance in male reproductive success (Waterman, 1998).

Shuster & Wade (2003) summarized predictions for the relationship between the spatial and temporal distribution of fertilizations and the opportunity for sexual selection. They predicted that when fertilisations are spatially clustered but temporally dispersed a small proportion of dominant males may be able to monopolise matings, with the consequence that variance in male reproductive success may be high. In contrast, when fertilisations are spatially dispersed but temporally clustered the opportunity for males to monopolise matings is limited and variance in male reproductive success is predicted to be low. Here we test the predictions of Shuster & Wade (2003) using European bitterling (*Rhodeus amarus*), hereafter referred to simply as bitterling. Bitterling are freshwater fishes that show an unusual spawning relationship with freshwater mussels (Smith et al., 2004). During the spawning season males develop bright colouration and defend territories around mussels. Females develop long ovipositors that they use to lay their eggs on the gills of a mussel through the mussel's exhalant siphon. Males actively court females and lead them to mussels in their territories. Females can only use mussels for oviposition, which they inspect before spawning, basing their spawning site choices on mussel and male quality (Smith et al., 2000, 2001; Mills & Reynolds, 2002; Kitamura, 2005, 2006; Smith & Reichard, 2005), with consistent preferences for certain mussel characteristics; mussels containing few bitterling eggs are preferred and *Unio* spp. mussels are used in preference over *Anodonta* spp. (Smith et al., 2002, 2004; Reichard et al., 2007), and for large colourful males that engage in high rates of courtship (Candolin & Reynolds, 2001; Smith et al., 2002, 2003; Reichard et al., 2005). Males fertilize the eggs by releasing sperm into the inhalant siphon of the mussel, so that water filtered by the mussel carries the sperm to the eggs. Males show alternative mating tactics and are able to behave as both territory holders and sneakers. These reproductive roles are flexible and all males may play either role or both; territorial males frequently participate in matings in the territories of rivals as sneakers (Kanoh, 1996, 2000; Smith et al., 2003; Reichard et al., 2004a,b, 2005; Smith & Reichard, 2005). Those males that control access to mussels enjoy high reproductive success (Kanoh, 1996, 2000; Reichard et al., 2004a,b). Embryos remain inside the host mussel for about one month before emerging as well-developed larvae (Aldridge, 1999; Smith et al., 2004).

Because bitterling must use mussels for reproduction it is possible to manipulate spawning site distribution, density and quality, making bitterling ideal models for understanding the role of the spatial and temporal distribution of fertilizations in mating system evolution (Smith et al., 2004, 2006). Here we use a laboratory and field experiment to investigate the effect of the abundance and quality of spawning sites on male and female spawning decisions and thereby how females distribute fertilizations among potential mating partners. Specifically, we tested the following predictions; (1) male bitterling show a preference for monopolizing clusters of oviposition sites rather than single oviposition sites, and prefer high over low quality oviposition sites and (2) females show a preference for spawning in clusters of oviposition sites, and prefer high- over low-quality oviposition sites. An outcome of predictions 1 and 2 will be that males that monopolize clusters of spawning sites could achieve a higher reproductive success than males guarding solitary spawning sites. Consequently, a final prediction is that the spawning rate will be greatest in situations when oviposition sites are clustered.

Methods

Selection of spawning sites in relation to number and quality of mussels: laboratory experiment

Data were collected during May 2004 in the aquarium facility at the University of Łodz, Poland. An experiment was conducted to investigate male and female oviposition choice in relation to 2 factors; mussel number and mussel quality. Approximately 150 bitterling were collected using a 5 m fine-mesh Seine net from Lake Kociolek, a small glacial lake (2.8 ha) in central Poland (52°37′02″N; 18°28′42″E), and transported in lake water to the aquarium at the University of Łodz. Fish were housed in four aquaria measuring 110 (length) × 50 (height) × 50 (width) cm under a natural light regime and fed frozen chironomid larvae and commercial fish flakes prior to and during experiments. Fish were housed in mixed sex groups; without mussels female bitterling are unable to spawn. The experimental design did not control for familiarity with mates, though spawning site quality appears to be the main determinant of female oviposition decisions (Smith et al., 2004; Casalini, 2007). Aquaria were aerated continuously and water quality was maintained using filters. Mussels for the experiment were collected by hand from the Sulejowski Reservoir, located on the River Pilica in April, before the start of the bitterling spawning season. For tests, two species of mussel were used; *Anodonta anatina* and *Unio pictorum*. European bitterling show a clear spawning preference for *U. pictorum* over *A. anatina* (Smith et al., 2004), and this preference matches embryo mortality rates, with higher embryo mortalities in *A. anatina* than *U. pictorum* (Smith et al., 2000). Thus, *U. pictorum* represented a higher-quality spawning site than *A. anatina*.

Experiments began three days after the fish were collected and were conducted in an aquarium measuring 110 (length) × 50 (height) × 50 (width) cm with a 10 cm deep sand substrate. A single male bitterling and two females that were ready to spawn (evident from their extended ovipositors) were haphazardly caught in holding aquaria and gently released in the test aquarium. Test mussels were added to the test aquarium in a predetermined random order of treatments in each of two sand-filled pots placed at each end of the aquarium, 100 cm apart. The mussel treatments were; four mussels of high quality (i.e., *U. pictorum*) and one mussel of low quality (*A. anatina*), four mussels of low quality and one mussel of high quality, four mussels of high quality and one mussel of high quality, four mussels of low quality and one mussel of low quality. Mussels in groups of four were placed in a square configuration directly adjacent to each other. Each treatment was replicated 5 times. *U. pictorum* and *A. anatina* were used because European bitterling encounter both species in the west of their distribution and show a clear preference for *U. pictorum*. Either a single or four mussels were used because this represents the upper and lower number of mussels that male bitterling have been observed to defend under natural conditions (Smith et al., 2004; C. Smith, pers. obser.). After placing mussels in pots they were initially covered with a pair of empty pots to prevent the bitterling approaching them. After 5 min the empty pots were removed and the behaviour of the fish was recorded for 10 min. The behaviours recorded were the position of the mussel in which females spawned, the frequency of sperm releases by the male, the position of the mussel to which the male led females, and the rate of 'deceptive female oviposition', in which a female performs a spawning action but without releasing eggs (Smith et al., 2004, 2007). Male ejaculatory behaviour is unambiguous and involves the male sweeping quickly down over the inhalant siphon of the mussel (Smith et al., 2004). Sperm density may vary among ejaculations (C. Pateman-Jones, pers. obser.), however sperm release was taken to represent a preference for an oviposition site. Deceptive female oviposition is a conspicuous behaviour and entails the female making contact or almost making contact with the mussel siphon with the base of her ovipositor, but without inserting the ovipositor into the mussel siphon or releasing eggs. Deceptive female oviposition 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., 2001, 2004, 2007). Notably, deceptive female oviposition closely mimics spawning behaviour; it attracts the attention of males, and is displayed at a significantly higher frequency by females exposed to more than a single male (Kanoh, 1996, 2000; Smith & Reichard, 2005; Smith et al., 2007). Consequently, this behaviour may function to signal to males a female's readiness to spawn in a mussel. For a full description of behaviours see Smith et al. (2004). Missed ovipositions, which are relatively rare, were not observed in the study. Following completion of a trial all the experimental subjects, fish and mussels, were removed and none were used in the experiment again.

Selection of spawning sites in relation to number and quality of mussels: field experiment

A field experiment was conducted during May 2004 in Lake Kociolek to investigate the distribution of bitterling eggs in mussels arranged either singly or in groups under natural conditions. Bitterling are abundant in the lake, yet the number of mussels is relatively low, which permitted control over bitterling access to spawning sites. Mussels for the experiment were collected by hand from the Sulejowski Reservoir before the start of the bitterling spawning season. Four species of mussel were used; *A. anatina*, *A. cygnea*, *U. pictorum* and *U. tumidus*. Mussels were arranged in 16 arenas spaced at approximately 20 m intervals around the margin of the lake, 1-3 m from the shore and in a water depth of approximately 0.5-1.5 m. At each arena five sand-filled pots were placed in an arrangement of four grouped together and a single pot sited 4 m away from the group. This arrangement of mussels was intended to allow a single territorial male to establish a territory around the group of four mussels and a second male around the single mussel. In pilot studies males always quickly established territories around mussels placed in the lake, and 4 m greatly exceeded the maximum distance between adjacent territories. A single mussel was placed in each pot; one each of the four test species in the group of four, and one of the test species in the solitary pot. The location of arenas in which each species of mussel was placed in the solitary pot was decided according to a randomly predetermined order. Thus, there were four treatments; group and solitary *A. anatina*, group and solitary *A. cygnea*, group and solitary *U. pictorum*, group and solitary *U. tumidus*, each treatment combination was replicated four times. As in the laboratory experiment, the *Anodonta* spp. represented low-quality spawning sites and *Unio* spp. high-quality sites (Smith et al., 2004). Four species, rather than just the two used in the laboratory experiment, were used to obtain a better idea of the interaction between mussel species preference and mussel number.

The mussels remained in position, exposed to spawning by bitterling, for 21 days. A period of 21 days is shorter than the minimum period for which bitterling embryos are incubated in mussels (Smith et al., 2004), but sufficiently long to enable a series of spawnings to occur. On 28 May 2004, all the mussels were collected and transported to the University of Łodź for dissection. Mussels were measured to the nearest 1 mm and all live bitterling embryos on the gills of mussels were counted.

Data analysis

All data were tested for normality using a Kolmogorov-Smirnov test and for equality of variance using a Bartlett's test. In the laboratory experiment a paired *t*-test was used to compare the effect of mussel grouping on male and female behaviour on the entire set of trials. A paired *t*-test was also used to compare mussel species preferences in the subset of trials in which the two mussel species were matched. In the field experiment, the total abundance of embryos in grouped and solitary mussel treatments was compared using a t-test. A two-way ANOVA was used to compare the total number of eggs and embryos per mussel in relation to grouping treatment and mussel species. The relationship between mussel length and number of eggs and embryos on their gills for each of the four species was tested using Pearson's correlation.

Results

Selection of spawning sites in relation to number and quality of mussels: laboratory experiment

A total of 20 choice tests using 100 mussels were completed with 20 different males and 40 females. The rate at which males led females to groups of four mussels was significantly higher than to solitary mussels (paired t-test, $t = 6.13$, df $= 19$, $p < 0.001$; Figure 1), and higher to *U. pictorum* than *A. anatina* (paired t-test, $t = 4.50$, df $= 9$, $p = 0.002$; Figure 1). The rate of ejaculation was also significantly higher over groups of mussels compared with solitary mussels (paired t-test, $t = 4.99$, df $= 19$, $p < 0.001$; Figure 1), and higher over *U. pictorum* than *A. anatina* (paired t-test, $t = 4.50$, df $= 9$, $p = 0.002$; Figure 1).

Females performed deceptive female oviposition behaviour over groups of mussels at a significantly higher rate than solitary mussels (paired t-test, $t = 4.00$, df $= 19$, $p = 0.001$; Figure 2) and over *U. pictorum* at a higher rate than over *A. anatina* (paired t-test, $t = 2.49$, df $= 9$, $p = 0.034$; Figure 2). The spawning rate of females was significantly higher in mussels arranged in groups than solitary (paired t-test, $t = 4.69$, df $= 19$, $p < 0.001$; Figure 2) and in *U. pictorum* than *A. anatina* (paired t-test, $t = 3.20$, df $= 9$, $p = 0.011$; Figure 2).

Selection of spawning sites in relation to number and quality of mussels: field experiment

All experimental mussels, with the exception of one *A. anatina*, were recovered from Lake Kociolek, yielding a total of 5630 bitterling eggs and embryos in their gill chambers. We detected a highly significant difference

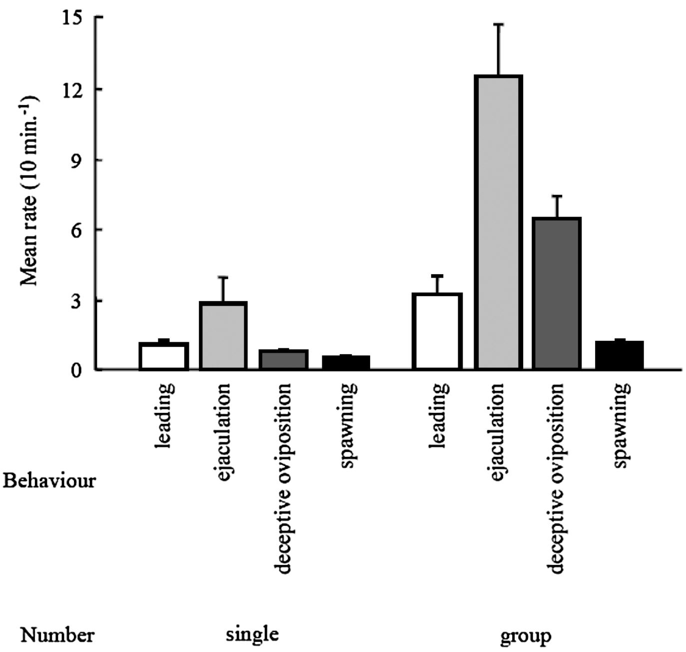

Figure 1. The mean rate of behaviours by territorial male and female bitterling (10 min^{-1}) with respect to whether mussels were single (low quality) or in groups of four (high quality). Errors bars are 1 standard error.

in the total number of eggs and embryos in mussels arranged in groups of four compared with solitary mussels (t-test, $t = 8.67$, df $= 29$ $p < 0.001$; Figure 3). However, there was no significant difference in the number of eggs and embryos *per* mussel in mussels in groups compared with solitary mussels (two-way ANOVA, data square-root transformed, $F = 0.30$, df $= 1, 63$, $p = 0.586$; Figure 4), though there was a significant effect of mussel species (two-way ANOVA, data square-root transformed, $F = 35.81$, df $= 3, 63$, $p < 0.001$), with significantly more eggs deposited on the gills of *Unio* spp. compared with *Anodonta* spp. (Tukey's test $p < 0.05$; Figure 4).

We detected a significant positive correlation between mussel total length and the number of eggs and embryos on the gills of *A. anatina* (Pearson's correlation, $r = 0.50$, df $= 33$, $p = 0.040$), but not for *A. cygnea* (Pearson's correlation, data $\log_{10}+1$ transformed, $r = 0.07$, df $= 35$, $p = 0.787$), *U. pictorum* ($r = -0.08$, df $= 35$, $p = 0.749$), or *U. tumidus* ($r = 0.42$, df $= 35$, $p = 0.082$).

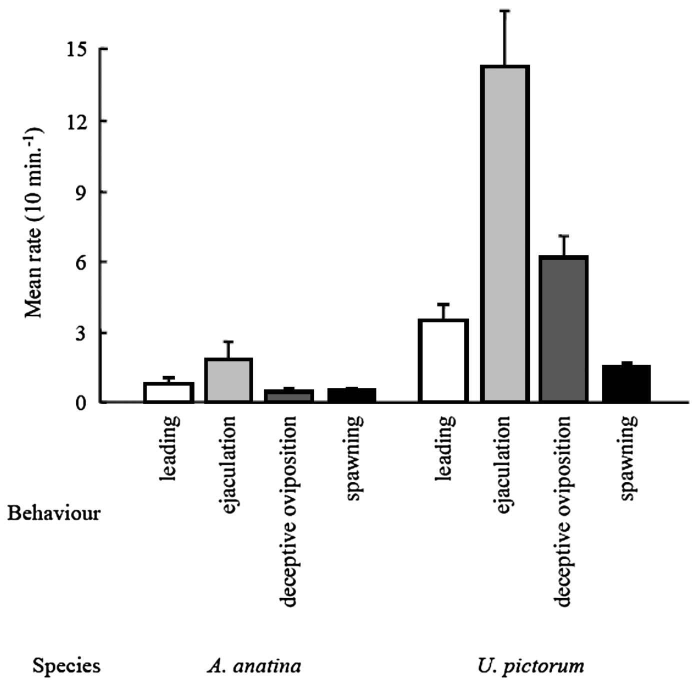

Figure 2. The mean rate of behaviours by territorial male and female bitterling (10 min^{-1}) with respect to mussel species; *A. anatina* (low quality) and *U. pictorum* (high quality). Errors bars are 1 standard error.

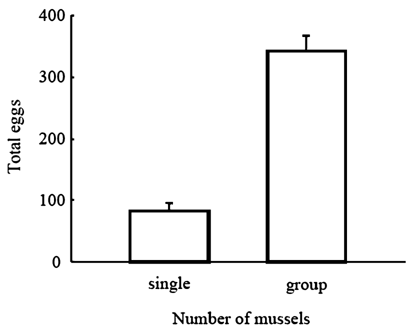

Figure 3. The mean number of eggs and embryos on the gills of single mussels and those arranged in groups of four over a 3-week period in Lake Wytopiskowe. Errors bars are 1 standard error.

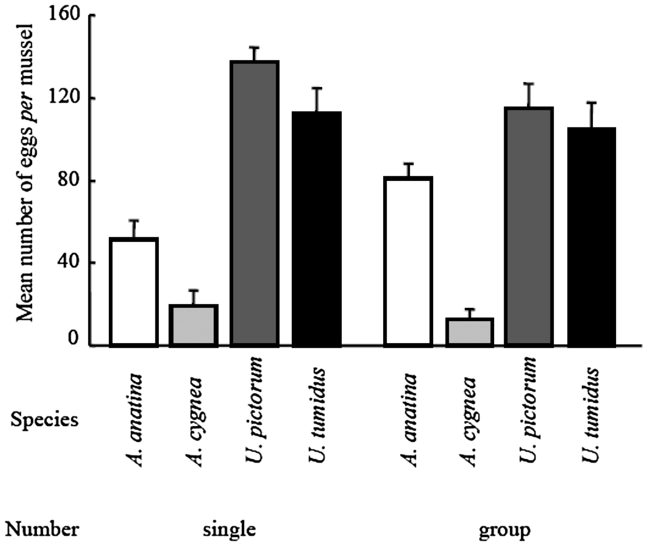

Figure 4. The mean number of eggs and embryos per mussel on the gills of single mussels and those arranged in groups of four species over a three-week period in Lake Kociolek. Errors bars are 1 standard error.

Discussion

Using the European bitterling, *Rhodeus amarus*, a fish that spawns on the gills of freshwater mussels, we investigated the effect of the distribution and quality of spawning sites on male and female spawning behaviour in a laboratory and field experiment. We showed that both males and females responded strongly to the number of spawning sites; males led females to mussels in clusters of four in preference to single mussels, and performed more ejaculations over groups of mussels. Females directed more spawning-related behaviour towards groups of mussels and laid eggs more frequently in territories with groups of mussels. In a field experiment, the total number of developing bitterling eggs and embryos recovered from territories containing groups of mussels was significantly higher than territories with single mussels, though the per capita number of eggs and embryos in mussels was equivalent irrespective of whether they were clustered or solitary.

These results imply that male bitterling that guard patches of mussels could achieve a higher reproductive success than those guarding solitary mussels. Females spawned preferentially in mussels when they were arranged in groups (laboratory experiment). When mussels received multiple spawnings a group of mussels accommodated more eggs and embryos than a solitary mussel (field experiment). Consequently, where mussels show a clumped rather than regular distribution, so that males are able to monopolize several mussels simultaneously, high variance in male reproductive success can be expected, which will tend to increase the opportunity for sexual selection in males, as predicted by Shuster & Wade (2003). This observation is supported by genetic and behavioural studies on bitterling by Reichard et al. (2004a,b). In their study increased male density had a comparatively modest effect on the strength of sexual selection, whereas when the spatial clustering of mussels was increased, variance in male reproductive success increased substantially. Thus, the abundance and spatial distribution of freshwater mussels may play a central role governing the reproductive success of male bitterling and thereby mating system evolution in this species. Our results raise the prospect that the opportunity for sexual selection may vary among bitterling population in relation to the abundance and distribution of mussels.

Spawning in bitterling frequently involves sneaked matings (Smith et al., 2002, 2003; Reichard et al., 2004a,b, 2005; Smith & Reichard, 2005), whereby non-territorial males and males in adjacent territories attempt to participate in a spawning in the territory of a rival male. Sneaky mating may have implications for the opportunity for selection, since this behaviour potentially allows males that might otherwise fail to mate to father some offspring, and thereby sneaking could reduce variance in male mating success. Reichard et al. (2004a) showed that although increasing the spatial clustering of mussels increased variance in male reproductive success when male density was low, at a high density variance was unchanged. This effect arose because at high densities male territorial defence of mussels broke down as a result of repeated incursions by sneakers, which eroded the reproductive success of territorial males (Reichard et al., 2004a). In the case of bitterling, clusters of mussels may attract more sneaker males than solitary mussels, which might erode any additional benefit to a territorial male of guarding a cluster of mussels. However, the opposite effect may also occur in some species, with sneaking increasing variance in male reproductive success. This situation arises if a small number of males are able to monopolize matings through a combination of courtship and sneaking. For example, Le Comber (2003) showed that in the three-spined stickleback, *Gasterosteus aculeatus*, it was brightly coloured males with territories that were the most successful both at attracting females and sneaking the fertilizations of neighbouring territory-holding males.

In our field experiment we found an equivalent number of bitterling eggs and embryos *per* mussel for each mussel species, irrespective of whether the mussels were arranged in groups or were solitary (Figure 4). This finding appears to contradict the significant behavioural preference for mussels arranged in groups demonstrated in the behavioural study. However, the result accords with a study by Smith et al. (2000), which showed that eggs were deposited in different mussel species by bitterling in relation to the density-dependent mortality rate of embryos in mussels. The distribution of eggs and embryos conformed to an ideal free distribution (Fretwell & Lucas, 1970), such that the quality of mussels, measured in terms of embryo survival, attained a common level among mussel species. Females inspect mussels before oviposition and make spawning decisions based on mussel quality, the principle determinants of which are mussel species and fullness with eggs and embryos (Smith et al., 2000). The oxygen content of water emerging from the exhalant siphon of a mussel is probably the cue used by females for making oviposition decisions with respect to mussel quality; certain species of mussel and those containing large numbers of eggs/embryos have a low oxygen content in the water emerging from the exhalant siphon and are avoided by bitterling (Smith et al., 2001, 2004). Thus, although groups of mussels appear to attract male and female bitterling, mussel quality (determined by species and fullness with eggs/embryos) governs whether spawning will occur. The result is that the number of eggs/embryos per mussel for a given species will be equivalent, though groups of mussels will accommodate more eggs/embryos in total than solitary mussels (Figure 3).

We detected evidence for an effect of mussel size on the number of eggs deposited on the gills of *A. anatina*. This effect has not been observed previously, and was relatively weak in the present study. Previous studies by us have failed to detect an effect of mussel size on bitterling oviposition decisions (Smith et al., 2000, 2001, 2004), and mussel size does not appear to be an important determinant of mussel choice by bitterling.

Our results have broader implications for mating system evolution. The intensity of sexual selection depends on the skew in reproductive success within and between the sexes (Emlen & Oring, 1977; Shuster & Wade, 2003), and many mating systems are characterized by high variation in the reproductive success among males, who are limited in their reproductive success by the number of mates they can attract (Andersson, 1994). Our results suggest that the quality and quantity of resources critical to reproduction can directly affect male reproductive rate; both of territorial males, but also of males adopting alternative mating tactics. This effect arises both from intra- and intersexual selection. Choice of oviposition site may be as important as choice of mate in determining female reproductive success in bitterling (Smith et al., 2000, 2003), but also in other taxa, such as pied flycatchers, *Ficedula hypoleuca* (Alatalo et al., 1986), spotted wrasse, *Notolabrus celidotus* (Jones, 1981) and bluehead wrasse, *Thalassoma bifasciatum* (Warner, 1987). Even in species where males provide care to offspring, female mate choice is often influenced by nest site characteristics, such as predation risk and prevailing oxygen conditions (Kraak et al., 2000; Payne et al., 2002). Further, the level to which fertilizations can be controlled by individual males is a function of the scarcity and patchiness of mating sites; relatively rare and highly clustered sites will be most defendable. Thus, variance in male reproductive success will also be underpinned both by intrasexual selection and environmental attributes (Emlen & Oring, 1977).

In summary, this study shows that male and female European bitterling have a spawning preference for mussels in groups over solitary mussels. Territories with groups of mussels were also shown to accommodate more eggs than those with single mussels. Consequently, the spatial arrangement of sites of reproduction and, therefore, of fertilisations, may directly affect variance in male reproductive rate and the opportunity for sexual selection, in accordance with the predictions of Shuster & Wade (2003).

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