Sex differences in the responses to oviposition-site cues by a fish revealed by tests with an artificial host

ABSTRACT

Oviposition decisions can have important fitness consequences for offspring. We investigated the responses of European bitterling (*Rhodeus amarus*), a freshwater fish that spawns in the gills of living unionid mussels, to oviposition-site cues. Using an artificial mussel we manipulated the flow velocity, dissolved oxygen concentration and odour cues of mussels presented to pairs of *R. amarus*. Females responded positively to mussel odour, and to dissolved oxygen cues. Male response was dependent on mussel odour and the flow velocity of water emerging from the artificial mussel. These responses are potentially adaptive, with females responding to cues that indicate the quality of oviposition sites for incubation of eggs. Males responded to cues with implications for optimal sperm allocation.

Keywords

maternal effect, mussel, oviposition-site cue, *Rhodeus*, reproduction, sexual conflict
Irrespective of mating system, mothers exert primary control over their propagules, either through the size and number of offspring, provisioning of eggs and embryos and, in taxa in which female mate choice operates, the paternal contribution of genes to offspring (Mousseau & Fox, 1998). In many taxa the female is the sole or predominant care-giver (Clutton-Brock, 1991). Maternal experience can also be transmitted to offspring through cytoplasmic factors that influence offspring development (Smith & Ritchie, 2013). An additional means by which a mother can contribute to offspring success is through her oviposition-site decisions (Mousseau & Fox, 1998; Roitberg, 1998; Refsnider & Janzen, 2010), particularly in species that oviposit on discrete patches of resource, such as parasitoids (Taylor et al., 1998), brood parasites (Soler, 2014), phytophagous insects (Mayhew, 1997), and seed beetles (Cope & Fox, 2003). Oviposition-site decisions have long been recognized as having significant evolutionary and ecological consequences (Adolph, 1920; Refsnider & Janzen, 2010).

Maternal oviposition-site decisions can affect the fitness of offspring if oviposition sites vary in quality. Quality may vary among resource types (e.g. among a range of host species), or because females 'superparasitise' a resource patch (i.e. they deposit their eggs on the same resource patch as other females). The immediate and longer-term fitness outcomes of maternal oviposition decisions have received attention (Shine & Harlow, 1996; Spence & Smith, 2013). In many taxa, however, males also play a role in oviposition decisions (Refsnider & Janzen, 2010).

The oviposition-site decisions of males and females need not correspond. In some mating systems males can influence female oviposition-site decisions through harassment (Córdoba-Aguilar, 2009), the transfer of ejaculatory substances during mating (Wolfner, 2002), and by controlling access to oviposition sites (Qvarnström & Forsgren, 1998), potentially resulting in sexual conflict (Spence & Smith, 2005). If oviposition-site decisions have different outcomes for the sexes and represent a possible arena for sexual conflict, a key question is whether the sexes attend to the same or different cues in making decisions.
Here we investigate the responses of male and female European bitterling (*Rhodeus amarus*) to oviposition-site cues. *R. amarus* are a small freshwater cyprinid fish that use living unionid mussels for oviposition (Smith et al., 2004). Males defend small territories focused on one or more freshwater mussels (Tinbergen, 1951; Wiepkema, 1961), to which they 'lead' females to spawn a clutch of 1-6 eggs. Females use a long ovipositor to place their eggs inside the gill cavity of a mussel and the male fertilizes the eggs by releasing sperm over the mussel (Smith et al., 2004). Bitterling embryos complete development inside the mussel gill, imposing significant costs on the host for their month-long development. Females lay approximately 250 eggs over the course of a single spawning season and, therefore, make multiple oviposition-site decisions (Smith et al., 2004). Spawning in bitterling often involves 'sneaky' matings, whereby a male that has not courted a female may successfully participate in spawning (Smith et al., 2015).

Developing bitterling eggs and embryos compete with the host for oxygen and nutrients (Spence & Smith, 2013), limiting mussel growth and fecundity (Reichard et al., 2006) and potentially damaging gill tissue (Stadnichenko & Stadnichenko, 1980). Multiple clutches can be deposited in the same mussel, and egg and embryo mortality is strongly density dependent (Smith et al., 2000; Spence & Smith, 2013). Different mussel species vary in quality as hosts, and bitterling are choosy about which mussels are used for oviposition (Smith et al., 2000; Casalini et al., 2013). Thus oviposition-site decisions are a key component of the bitterling mating system, with immediate (Smith et al., 2000; Mills & Reynolds, 2002a) and longer-term (Agbali & Smith, 2012) consequences for fitness.

The cues used by bitterling for oviposition-site decisions are ambiguous. *R. amarus* show a response to water flow from the exhalant siphon of a mussel (Smith et al., 2001; Mills & Reynolds, 2002b), but also to the dissolved oxygen concentration of the exhalant flow (Smith et al., 2000, 2001). Separating the effects of these two cues is problematic because flow velocity and dissolved oxygen concentration are potentially correlated (Davenport & Woolmington, 1982). Additionally, there is evidence that visual and odour cues and the presence and behaviour...
of other bitterling can influence oviposition choice (Heschl, 1989; Smith & Reichard, 2005). Males and females may not express the same host preferences. Casalini et al. (2013) suggested that males tracked female host preferences and it may be the case that male behaviour does not directly indicate preference for a mussel, but instead represents adaptive plastic behaviour towards a host. Here we experimentally investigated the strength of response of \emph{R. amarus} to oviposition site cues. We examined three discrete cues; water flow velocity, dissolved oxygen concentration, and mussel odour, using an artificial mussel that permitted us to manipulate each cue independently. We addressed the question of whether single or multiple cues are used and whether males and females use the same or different cues.

**METHODS**

\emph{General methods}

Approximately 350 \emph{R. amarus} were collected from a river at the centre of the distribution of the fish in Europe. In addition, approximately 180 \emph{Unio tumidus} mussels were collected from an adjacent oxbow lake (where both bitterling and mussels are abundant) prior to the start of the spawning season during April 2015. Fish and mussels were transported to outdoor fiberglass tubs (1.3 x 1.3 m). Each tub was filled to a depth of 0.6 m with 1000 litre of water that had been left to dechlorinate for three days and furnished with a gravel substrate and artificial plants as refuges. Fish were stored in mixed sex groups at low densities (approximately 30 fish per tub) and fed \textit{ad libitum} three times daily with a mixture of frozen chironomid larvae and copepods. Mussels were stored separately from fish. Approximately one third of the water in tubs containing fish and mussels was changed twice weekly to maintain water quality. Given the low densities of bitterling, which are small fish (typically < 60 mm standard length), poor water quality was not a problem during the study. Fish and mussels in tubs were exposed to natural light and temperature variation, typical for mid-May in central Europe. Mean (± SD) water temperature was 17.9 (±
2.5) °C, and there were approximately 15.5 h daylight hours each day over the experimental period.

Experiments were conducted in fiberglass tubs identical to those used to store fish and mussels. Eight experimental tubs were stocked with three male *R. amarus* and a *U. tumidus* mussel in a sand-filled plastic pot. The pot kept mussels in a fixed position while permitting them to adopt a natural orientation. Males were left for at least 24 h to settle before the start of the experiment. In each case one male (always the largest) established dominance in the experimental tubs and actively guarded the mussel. This individual served as the focal male in the experiment. Non-focal males occasionally inspected the experimental mussel when the focal male was not present, but did not participate in spawning behaviour with the focal female. While these non-focal males served to encourage guarding and territoriality by the focal male, any effects they might have had on the focal pair did not vary among experimental treatments and their presence simply served to make the experimental set up comparable with natural conditions.

To start the experiment a female with an extended ovipositor, indicating a readiness to spawn, was gently caught in one of the stock tubs and transferred to a glass box measuring 220 (height) x 80 (width) x 80 (depth) mm with a mesh top to permit water exchange. The female was placed in a pre-selected experimental tub 300 mm from the mussel guarded by the focal male. Once the focal male began courtship and the female showed a response by attempting to follow him, the live *U. tumidus* was replaced with an artificial mussel and the female was released from the glass box. Any odour from the live mussel was diluted quickly within the 1000 litre experimental tub and so would not have affected behaviour towards the artificial mussel. This experimental design was intended to accommodate the mating system of European bitterling. In nature, males are highly territorial around a patch of mussels, while females display no site attachment and range among male territories, feeding and spawning over an extensive area (Smith et al., 2004). Thus the design we used, with males confined to a territory, and gravid females gently introduced to these territories for short intervals, mirrored natural conditions.
Artificial mussels comprised a 35 mm plastic film canister measuring 50 (length) x 30 (diameter) mm with a snap on lid. The lid of the canister had two openings; an exhalant aperture of 10 mm and an inhalant aperture of 5 mm. The female bitterling releases eggs through the exhalant siphon while the male releases sperm over the inhalant siphon. A Venturi system generated an exhalant and inhalant flow. Water flowed into the base of the artificial mussel under gravity, through a constriction to elevate flow velocity, and out of the exhalant aperture (Figure 1). The elevated water flow velocity and reduced static pressure generated by the constriction created an inward flow of water through the inhalant aperture of the artificial mussel (Figure 1). This design of artificial mussel permitted the source and rate of flow to be experimentally manipulated. In pilot studies males guarded artificial mussels, led females to them to spawn and ejaculated over the inhalant aperture. Similarly, females inspected the exhalant aperture of artificial mussels and spawned in them. We detected no negative effects of potential endocrine-disrupting chemicals derived from artificial mussels. Even if present, the dilution of these chemicals in experimental tubs, combined with the extremely short time to which fish were exposed to them, meant that the reproductive system of experimental fish was unlikely to have been compromised.

*R. amarus* were exposed to all combinations of three experimental mussel treatments; high and low flow rate, high and low dissolved oxygen concentration, and the presence and absence of mussel odour (Table 1), thereby generating eight treatment combinations (Table 2). Treatment combinations were imposed in a predetermined random pattern and a total of 80 experimental trials were conducted over the study, with 10 replicates of each treatment combination.

Artificial mussels were connected by 5 mm diameter PVC tubing to a 1000 litre reservoir ('source tub') that was raised approximately 0.6 m above the level of the experimental tub in which observations were carried out. To create a high dissolved oxygen concentration (DO), water in the reservoir was strongly aerated with an air pump. To create a low dissolved oxygen concentration, nitrogen was bubbled through water in the reservoir. Dissolved oxygen
concentration was monitored with a dissolved oxygen meter (HORIBA U-222). Mean ± 95% CI dissolved oxygen concentration in high oxygen treatment reservoirs was 7.48 ± 0.21 mg O\textsubscript{2} /litre, and low treatment 1.48 ± 0.14 mg O\textsubscript{2} /litre (Table 1). Algal growth in experimental tubs resulted in elevated dissolved oxygen concentrations through photosynthesis compared to source tubs, which were free of algae. The outcome was a higher ambient dissolved oxygen level in experimental tubs than in the water emerging from artificial mussel siphons, even in the high dissolved oxygen treatment (mean ± 95% CI high dissolved oxygen treatment 10.24 ± 0.28 mg O\textsubscript{2} /litre, low treatment 9.87 ± 0.25 mg O\textsubscript{2} /litre). Under natural conditions mussels consume between 7% and 90% (Smith et al. 2001) of oxygen flowing over their gills, depending on species, gravidity and parasitism by bitterling (Smith et al., 2000, 2001, Reichard et al., 2007a). Therefore, the dissolved oxygen concentration of water emerging from the siphons of artificial mussels, which declined to between 73% and 15% of the concentration of the surrounding water, accurately reflected the range naturally encountered by bitterling. To accommodate this feature of the study in our analysis, the difference in the ambient dissolved oxygen concentration in experimental tubs and the source tub supplying water to the artificial mussel was calculated and used as an additional covariate (see below). The mean ± 95% CI difference in dissolved oxygen concentration in the high oxygen treatment was 2.77 ± 0.26 mg O\textsubscript{2} /litre, and low treatment 8.40 ± 0.24 mg O\textsubscript{2} /litre. The volume of water flowing into the experimental tubs from the artificial mussels during observations (a maximum of 3 litre) was too low to have a measurable impact on oxygen conditions inside the experimental tubs (containing 1000 litre).

High and low flow rates were obtained by clamping the tube connecting the artificial mussel to the water source until the desired flow rate was achieved. The presence of mussel odour was achieved by placing 80 U. tumidus mussels in the 1000 litre source reservoir, a procedure previously used to elicit oviposition behaviour in R. amarus (Heschl 1989). Water quality was maintained with twice weekly water changes of approximately 250 litre of water and mussels were fed daily with phytoplankton. Mussels filter water at a rate of about 2 litres /hour (Smith et
al., 2001), hence the entire contents of the source reservoir would pass across the gills of the stocked mussels several times in 24 h. The source tubs were stocked with mussels two weeks prior to the start of the experiment to ensure a maximum concentration of odour was reached and pilot trials showed that bitterling were responsive to this concentration of odour. Any mussel odour cues in the small quantities of water transferred to experimental tubs when fish were moved (approximately 1 litre) would be rapidly diluted.

After replacing the live mussel with an artificial mussel the behaviour of the female and focal male was observed for 10 min. or until a spawning occurred. Behaviours recorded were, for the male: inspection of the exhalant aperture and ejaculation over the inhalant aperture (see Wiepkema, 1961 for full description). In females a record was made of inspection of the exhalant aperture and skimming, whereby the female sweeps quickly over the exhalant aperture, which she touches with the base of her ovipositor but without inserting her ovipositor into the mussel or releasing any eggs. Skimming behaviour encourages males to release sperm, and may function in assuring fertilisation of eggs (Smith & Reichard, 2005). Skimming has been proposed as a proxy for female mussel preference (Wiepkema, 1961; Candolin & Reynolds, 2001). Only one spawning occurred during observations, possibly due to the imperfect replication of a living mussel with an artificial one. Consequently, oviposition was not a suitable response variable for analysis, and skimming behaviour was instead used as a measure of female mussel preference.

After completion of observations the dissolved oxygen concentration and temperature of the experimental tub were measured and the female and focal male were captured and measured (standard length, to the nearest 1 mm). Fish were not used again in the study. After completion of the study all fish and mussels were returned to the sites from which they were originally collected. A total of eight artificial mussels were used in the study. Individual artificial mussels were randomized among treatments. A total of 80 experimental trials were conducted over the study, with 10 replicates of each treatment combination.

Statistical analysis
Prior to applying statistical models, a data exploration was carried out (Ieno & Zuur 2015). Homogeneity and zero inflation in the response variable were examined and collinearity between explanatory variables was investigated using variation inflation factors. Outliers in the data were identified visually using Cleveland plots. Male mussel inspection behaviour was found to be collinear with ejaculation frequency. Male inspection behaviour was subsequently dropped from the analysis, since sperm release over a mussel was taken to indicate an investment in a particular mussel and to better represent male mussel preference. Similarly, female mussel inspection behaviour, which was collinear with skimming, was dropped from the analysis. Models were fitted to data for male response (ejaculation frequency) and female response (skimming frequency). Because males and females could potentially influence the oviposition preferences of the opposite sex, we included the response variables of the opposite sex, along with experimentally manipulated mussel cues, as covariates when fitting the models. Male and female response variables were not collinear.

The data contained a high incidence of zero counts (50% for ejaculation frequency, 80% for skimming behaviour), though with responses distributed equitably among treatment combinations. Consequently, zero-altered (hurdle) models with Poisson (ZAP) or negative binomial (ZANB) distributions were employed (Zuur et al., 2009) using the pscl package ver. 1.4.6 (Jackman, 2014) in the R statistical environment, ver. 3.3.2 (R Development Core Team, 2016). Zero-altered models are partitioned into two parts, with a binary process modelling zeros and positive counts, and a second process modelling only positive counts using a zero-truncated model (Hilbe, 2014). This modelling approach enabled us to separately identify the mussel cues that elicited the occurrence of a behaviour (binary part), and the frequency of that behaviour when it occurred (zero-truncated part). For males a ZANB model was fitted as:

\[ e_{j,1} \sim ZANB(\mu_i, \pi_i, k) \]

\[ E(e_{j,1}) = \frac{1 - \pi_i}{1 - P_0} \times \mu_i \text{ where } P_0 = \left( \frac{k}{\mu_i + k} \right)^k \]

\[ \text{var}(e_{j,1}) = \frac{1 - \pi_i}{1 - P_0} \times (\mu_i + \mu_i^2 + \frac{\mu_i^2}{k}) - \left( \frac{1 - \pi_i}{1 - P_0} \times \mu_i \right)^2 \]
\[\log(\mu_i) = flow_i + muss_i + oxy_i + oxydiff_i + msl_i + fsl_i + temp_i + tub_i + skim_i\]

\[\logit(\pi_i) = flow_i + muss_i + oxy_i + oxydiff_i + msl_i + fsl_i + temp_i + tub_i + skim_i\]

Where \(ejac_i\) is the number of ejaculations by focal males in observation \(i\) assuming a negative binomial distribution with mean \(\mu\), probability \(\pi\) and dispersion \(k\) (Zuur et al., 2009). The variables \(flow_i\), \(muss_i\) and \(oxy_i\) are categorical covariates with two levels corresponding with artificial mussel water flow, mussel odour and dissolved oxygen, respectively. The variables \(oxydiff_i\), \(msl_i\), \(fsl_i\), and \(temp_i\) are continuous covariates corresponding with difference in dissolved oxygen concentration between artificial mussel and experimental tub (mg/litre), male standard length (mm), female standard length (mm) and water temperature of experimental tub (°C), respectively. The variable \(tub_i\) was included to control for an effect of experimental tub and \(skim_i\) was a continuous covariate that corresponded with female skimming frequency and was included to accommodate the effect of female behaviour on male mussel preferences.

For females a ZAP model was fitted as:

\[skim_i \sim ZAP(\mu_i, \pi_i)\]

\[E(skim_i) = \frac{\frac{1-\pi_i}{1-e^{-\mu_i}} \times \mu_i}{1 - e^{-\mu_i}}\]

\[var(skim_i) = \frac{1-\pi_i}{1-e^{-\mu_i}} \times (\mu_i + \mu_i^2) - \left(\frac{1-\pi_i}{1-e^{-\mu_i}} \times \mu_i\right)^2\]

\[\log(\mu_i) = flow_i + muss_i + oxy_i + oxydiff_i + msl_i + fsl_i + temp_i + tub_i + ejac_i\]

\[\logit(\pi_i) = flow_i + muss_i + oxy_i + oxydiff_i + msl_i + fsl_i + temp_i + tub_i + ejac_i\]

Where \(skim_i\) is the number of skims by focal females in observation \(i\) assuming a Poisson distribution with mean \(\mu\) and probability \(\pi\) (Zuur et al., 2009). The covariate \(ejac_i\) was included to accommodate the effect of male behaviour on female mussel preferences.

Best-fit zero-altered models were selected based on second-order Akaike’s information criterion (AICc; Akaike, 1973) using the AICcmodavg package ver. 2.1-0 (Mazerolle, 2016) by removing predictor variables from the full models until the model with the lowest AICc values were identified. To assess the robustness of each model we simulated 1000 datasets from the
best-fitting models and compared these with observed data, using the procedure of Zuur & Ieno (2016) for hurdle models.

Ethical Note

The experimental protocol was non-invasive, involving minimal handling of experimental fish (transfer to experimental tubs and length measurement) and optimal housing and experimental conditions (low density, multiple refuges, water changes twice weekly, *ad lib.* feeding). Fish were collected by electrofishing. We used a specially designed battery-driven pulse DC apparatus (Lena, Bednář Olomouc, Czech Republic), with a small diameter anode that selectively targeted fish smaller than 100 mm. Electrofishing was considered the least stressful method of capture (Janáč 2009), with much lower impacts on non-target stream biota than Seine netting, which involves indiscriminate capture and abrasive damage to fish. At the end of the study all bitterling and mussels were returned to their original sites of collection.

RESULTS

The presence of mussel odour cues was essential for determining whether males responded to a mussel with ejaculations (Figure 2, Table 3). In the presence of odour cues, the frequency of ejaculation was positively associated with high water flow velocity but negatively with male size (Figure 2, Table 3). Similarly, the presence of both mussel odour cues and a high dissolved oxygen concentration was needed for eliciting female skimming behaviour over artificial mussels (Figure 3, Table 3). In the presence of these cues, the frequency of skimming by females was positively related to the magnitude of the difference between ambient dissolved oxygen in experimental tubs and that emerging from the artificial mussel siphon (Figure 3, Table 3). There was also a negative association between female skimming frequency and focal male size (Figure 3, Table 3). Simulated data generated from our best-fit models generated distributions that complied with observed data.
DISCUSSION

The aim of this study was to identify the cues used by *R. amarus* in responding to oviposition sites. Appropriate responses to host cues are a key component of the mating system of this species (Smith et al., 2004), as well as other taxa (Refsnider & Janzen, 2010). We discriminated which cues were responsible for the occurrence of a response to a cue, and when a response did occur, its magnitude by fitting zero-altered statistical models (Zuur et al., 2009; Hilbe, 2014).

Both sexes expressed a positive response to water conditioned with the odour of living mussels; without this cue the reaction to artificial mussels was negligible. This response ensures that time and energy are only invested in living mussels, not water flows originating from some other source. Additional information may also be obtained from mussel odour cues. While *R. amarus* are generalists, potentially using a range of mussel species for oviposition, other bitterling species are specialists, using just one or two (Liu et al., 2006; Kitamura et al., 2012). In these cases, species-specific odour cues may play a role in mussel choice (Reichard et al., 2007a) as bitterling appear not to attend to visual cues that discriminate mussel species (Mills & Reynolds, 2002b).

Chemosensory cues are crucial in the oviposition-site decisions in other taxa, including *Drosophila* spp. (Riffell, 2013), fig wasps (Hossaert-McKey et al., 1994), mosquitos (Afify & Galizia, 2015) and parasitoids (Godfray, 1994). In *D. melanogaster*, research on the mechanistic basis to oviposition-site decisions has demonstrated a role for specific volatile compounds that activate specific neurons expressing a specific odorant receptor; thus a single dedicated olfactory pathway determines oviposition choice in this species (Dweck et al., 2013). An understanding of the mechanistic basis of a response to mussel odour by bitterling may provide insights into interspecific variation in host specialism in these fishes and artificial mussel is an ideal tool to achieve this goal.

Females showed a significant response to a high dissolved oxygen concentration. Oxygen availability is critical to egg and embryo development and survival during incubation in the mussel gill. Bitterling eggs are relatively large compared to other similarly sized fish, allowing
them to fit in the interlamellar spaces of a mussel gill, and consequently have a high per capita oxygen requirement (Aldridge, 1999). Given that mussels sometimes host well over 100 bitterling eggs (Smith et al., 2001; Kitamura, 2005), competition for oxygen inside the mussel gill can be severe, both among embryos and between embryos and host, and it is notable that embryo mortality rates in mussels are strongly density dependent (Smith et al., 2000, 2001; Agbali & Smith, 2012; Spence & Smith, 2013), presumably due to asphyxiation (Aldridge, 1999; Kitamura, 2006). Consequently, natural selection is predicted to favour a preference for cues that indicate directly whether a mussel is hosting the eggs and embryos of other females, or indirectly through the decline in quality of a mussel as a result of superparasitism. Thus the response by females for mussels with high concentrations of dissolved oxygen in the exhalant flow of the artificial mussel appears adaptive, indicating to a female a mussel in good condition that contains few other embryos, which are potential competitors of her own offspring. Such avoidance of superparasitism is particularly well understood in parasitoids (Godfray, 1994; Gandon et al., 2006) where in some wasps, females make oviposition decisions associated with interspecific, intraspecific and self-superparasitism via 'patch marking', chemical cues left by females during oviposition (van der Hoeven & Hemerick, 1990; Viser, 1993; Harvey, 2000). Whether female bitterling can detect bitterling eggs and embryos in mussel gills is not currently known, however the indirect detection of superparasitism from dissolved oxygen levels in the mussel exhalent flow may operate in the bitterling system.

While female R. amarus showed a significant threshold response to high dissolved oxygen conditions, the strength of female response to artificial mussels was predicted by the difference between the dissolved oxygen concentration of the mussel exhalant flow and the ambient oxygen concentration; the lower the exhalant flow dissolved oxygen concentration relative to ambient, the lower the female response (Table 3). The implication of this finding is that the female response to an oxygen cue is labile and based on comparative evaluation, rather than a fixed response to a threshold dissolved oxygen concentration. This outcome fits in the context of the
ecology of bitterling and their mating system. The spawning season of *R. amarus* is relatively protracted, starting in mid-April and typically ending in mid-June (Konečná & Reichard, 2011). As the spawning season advances, mussels fill with eggs and embryos and progressively decline in quality as incubation sites (Kitamura, 2005; Smith, 2017). Water temperatures also progressively increase, with a concomitant decline in dissolved oxygen concentration. The capacity to distinguish the relative, rather than absolute, quality of an individual mussel is, therefore, critical in accommodating this temporal pattern in oviposition site quality based on dissolved oxygen conditions inside the mussel.

Dissolved oxygen availability plays a key role in the oviposition decisions of a number of other fish species (Wootton & Smith, 2015). For example, in beaugregory damselfish (*Stegastes leucostictus*) the rate of development and survival of embryos are dependent on oxygen availability, and spawning sites are selected on this basis, with dissolved oxygen as a cue (Payne et al., 2002). In salmonids, females assess substrate quality and hyporheic flow prior to preparing spawning redds and oviposition (Chapman, 1988; Bernier-Bourgault & Magnan, 2002; Brabrand et al., 2002; Esteve, 2005).

Male *R. amarus* responded to water flow velocity from artificial mussels with an elevated ejaculation frequency. The approach taken in this study does not allow the framing of this preference as “choice” by the males. Males here displayed a plastic response to flow by not exclusively ejaculating in high flow mussels, but only in adjustments to their behaviour in response to flow. This positive, plastic response by males to water flow may reflect an unusual aspect of the bitterling mating system. Male *R. amarus* perform multiple ejaculations over mussels, even without a female present, ejaculating over a guarded mussel on >200 occasions daily under natural conditions (Smith et al., 2009). This pattern of sperm release appears to function in maintaining a baseline level of spermatozoa in a mussel's gills (Smith & Reichard, 2013), thereby ensuring fertilization should a female oviposit in the mussel. Sperm released into a mussel potentially undergoes passive loss from its gills as it filters water. The rate that males
‘top-up’ mussels with sperm differs between bitterling species, and is sensitive to the presence of
rivals and females in spawning condition (Smith et al., 2014a). Filtration rates vary naturally
among (Smith et al., 2001) and within host mussel species (Mills & Reynolds, 2002b). Smith &
Reichard (2013) speculated that because mussels filter water at different rates (either due to
species or individual differences) males might be sensitive to mussel flow rate and should
respond to elevated flow rates by increasing ejaculation rates to keep mussels topped-up with
sperm (sensu Parker, 1998). The results of the present study support this hypothesis (Figure 3).
Thus while variation in mussel flow rates did not inhibit male host preference, our results
demonstrate that males are capable of adjusting their behaviour adaptively to their current host.

Male size was negatively associated with the frequency of ejaculation and also female
response to mussels (Table 3). Male size determines dominance in bitterling (Smith et al., 2003;
Casalini et al., 2009), with the largest males tending to act as guarders and smaller males acting
as sneaks (Smith et al., 2004). This pattern is a common feature of the mating systems of fishes
(Wootton & Smith, 2015) and other taxa (Arnqvist & Rowe, 2005). Smaller male bitterling have
relatively (though not absolutely) larger testis size (Smith et al., 2014a) and typically compete
with rivals through sperm competition rather than direct aggressive contests (Reichard et al.,
2004), which may explain the higher ejaculation rate of smaller males in the present study. Male
bitterling increase their sperm investment through elevated frequency of ejaculation, not larger
ejaculate size (Candolin & Reynolds, 2002). The reason for a greater female response to smaller
males is unclear. Male size and dominance do not appear to play a role in female mate choice,
though large dominant males are typically able to monopolize mussels and thereby to achieve
high reproductive success (Reichard et al., 2007b, 2009; Casalini et al., 2009). Male nuptial
colour similarly has not been demonstrated to have a direct effect on female mate choice
(Reichard et al., 2005; Casalini et al., 2009). Without measuring further male traits such as
 genetic compatibility, we are unable to account for this apparent elevated response by females to
smaller males.
Overall our results demonstrated that males and females responded to common, but also contrasting mussel cues. Both sexes responded almost exclusively to artificial mussels with the odour of living mussels, but while males failed to respond to dissolved oxygen levels, females showed a response to a high dissolved oxygen concentration and large relative difference in oxygen concentration between the artificial mussel and ambient. In contrast, while females did not respond to differences in water flow from the artificial mussel, males responded to higher flows by elevating their ejaculation rate (Table 3). These differences may reflect different adaptive priorities for males and females. Thus, while females attend to cues that reflect mussel quality as a site for incubation of young stages (Smith et al., 2001, 2002; Agbali et al., 2010; Agbali & Smith, 2012), males instead appear sensitive to the risk of sperm competition (Spence, Reichard & Smith, 2013), and are insensitive to mussel quality (Smith et al., 2002, 2003, 2014b; Casalini et al., 2013). A sexual conflict over responses to oviposition sites in *R. amarus* may, consequently, arise. Sexual conflict occurs when the evolutionary interests of individuals of the two sexes diverge (Parker, 1979), with a potential to generate sexually antagonistic selection (Lessells, 2006). In the context of the bitterling mating system, responses to oviposition-site cues are a potential arena for sexual conflict, with females maximizing offspring fitness through attending to the dissolved oxygen concentration of water emerging from the mussel exhalant siphon, and males maximizing fertilization success through sperm competition by responding to water flow velocity and the behaviour of rivals. These differences appear to manifest themselves as overt behavioural conflicts between spawning partners (Smith et al., 2002). Over the course of a spawning event males repeatedly attempt to lead females away from mussels with nearby rivals while females frequently select alternative mussels on the basis of offspring survival. We are aware of no other mating system with conflicting responses to oviposition-site cues like that seen in *R. amarus*. 
REFERENCES


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Table 1. Experimental artificial mussel treatment combinations used in the study.

<table>
<thead>
<tr>
<th>Mussel odour</th>
<th>Dissolved oxygen</th>
<th>Flow velocity</th>
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<tbody>
<tr>
<td>Present</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>Low</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>High</td>
<td>Absent</td>
<td>Low</td>
</tr>
<tr>
<td>Absent</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>Low</td>
</tr>
</tbody>
</table>
Table 2. Experimental artificial treatments to which European bitterling were exposed in trials.

<table>
<thead>
<tr>
<th>Cue</th>
<th>Treatment</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow velocity</td>
<td>High</td>
<td>300 ml/min</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>100 ml/min</td>
</tr>
<tr>
<td>Dissolved oxygen</td>
<td>High</td>
<td>7.5 mg/l</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>1.5 mg/l</td>
</tr>
<tr>
<td>Mussel odour</td>
<td>Present</td>
<td>80 mussels/m³</td>
</tr>
<tr>
<td></td>
<td>Absent</td>
<td>0 mussels/m³</td>
</tr>
</tbody>
</table>
Table 3. Results of best-fit zero-altered negative binomial (ZANB)\textsuperscript{a} and zero-altered Poisson (ZAP)\textsuperscript{b} models testing the effects of mussel parameters on the responses of male and female *R. amarus*. Bold text indicates significant results to P < 0.05.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Parameter</th>
<th>Occurrence model</th>
<th>Frequency model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>γ</td>
<td>SE</td>
<td>Z</td>
</tr>
<tr>
<td>Male</td>
<td>Intercept</td>
<td>-1.34</td>
<td>2.99</td>
</tr>
<tr>
<td></td>
<td>Flow\textsubscript{(high)}</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Mussel\textsubscript{(present)}</td>
<td>1.99</td>
<td>0.52</td>
</tr>
<tr>
<td></td>
<td>DO\textsubscript{(high)}</td>
<td>0.91</td>
<td>0.52</td>
</tr>
<tr>
<td></td>
<td>Male size</td>
<td>-0.01</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>Skimming</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Female</td>
<td>Intercept</td>
<td>-1.95</td>
<td>3.94</td>
</tr>
<tr>
<td></td>
<td>Flow\textsubscript{(high)}</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Mussel\textsubscript{(present)}</td>
<td>3.28</td>
<td>1.09</td>
</tr>
<tr>
<td></td>
<td>DO\textsubscript{(high)}</td>
<td>1.50</td>
<td>0.70</td>
</tr>
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<td></td>
<td>O\textsubscript{2} difference</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Male size</td>
<td>-0.06</td>
<td>0.09</td>
</tr>
</tbody>
</table>
Figure 1
Cross-sectional assembly of artificial mussels used in the study. Arrows indicate direction of water flow.

Figure 2
Ejaculation frequency (over 10 minutes) by focal male R. amarus over an artificial mussel with and without mussel odour cues and low and high flow velocity against focal male standard length (mm) modelled using a zero-altered negative binomial (ZANB) model. Black circles are observed data.

Figure 3
Skimming frequency (over 10 minutes) by focal females over an artificial mussel with and without mussel odour cues and low and high dissolved oxygen concentrations in the exhalant flow against focal male standard length (mm) modelled using a zero-altered Poisson (ZAP) model. Black circles are observed data.
Fig. 1

Gravity-fed flow

Exhalant flow

Inhalant flow

10 mm
Ejaculation frequency (per 10 min)

Mussel odour absent

Mussel odour present

Low dissolved oxygen

High dissolved oxygen

Focal male standard length (mm)