



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



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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.

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Irrespective of mating system, mothers exert primary control over their propagules, through the size and number of offspring, provision 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; Refsnider & Janzen, 2010; Roitberg, 1998), particularly in species that oviposit on discrete patches of a resource, such as parasitoids (Taylor, Müller, & Godfray, 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

between resource types (e.g. between a range of host species), or because 'superparasitize' 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 some 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 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 investigated the responses of male and female European bitterling, *Rhodeus amarus*, to oviposition site cues. The bitterling is a small freshwater cyprinid fish that uses living unionid mussels for oviposition (Smith, Reichard, Jurajda, & Przybylski, 2004). Males defend small territories focused on one or more freshwater mussels (Tinbergen, 1951; Wiepkema, 1961), to which

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they 'lead' females to spawn a clutch of one to six 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 it (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, Phillips, & Reichard, 2015). Developing bitterling eggs and embryos compete with the host for oxygen and nutrients (Spence & Smith, 2013), limiting mussel growth and fecundity (Reichard, Ondračková, Przybylski, Liu, & Smith, 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, Reynolds, Sutherland, & Jurajda, 2000; Spence & Smith, 2013). Different mussel species vary in quality as hosts, and bitterling are choosy about which mussels are used for oviposition (Casalini, Reichard, Phillips, & Smith, 2013; Smith et al., 2000). 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. Bitterling show a response to water flow from the exhalant siphon of a mussel (Mills & Reynolds, 2002b; Smith, Rippon, Douglas, & Jurajda, 2001), 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 *R. amarus* to oviposition site cues. We examined three discrete cues, i.e. water flow velocity, dissolved oxygen concentration and mussel odour, using an artificial mussel that permitted us to manipulate each cue independently. We addressed the questions of whether single or multiple cues are used and whether males and females use the same or different cues.

METHODS

General Methods

Approximately 350 *R. amarus* were collected from a river at the centre of the distribution of the fish in Europe. In addition, approximately 180 *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 fibreglass tubs (1.3 × 1.3 m). Each tub was filled to a depth of 0.6 m with 1000 litres of water that had been left to dechlorinate for 3 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 *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 \pm 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 fibreglass 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. Nonfocal 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 nonfocal males served to encourage guarding and territoriality by the focal male, any effects they might have had on the focal pair did not vary between 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 80 × 80 mm and 220 mm high with a mesh top to permit water exchange. The female was placed in a preselected experimental tub 300 mm from the mussel guarded by the focal male. Once the focal male began courtship and the female responded 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 50 mm long and 30 mm in diameter 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 (Fig. 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 (Fig. 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 the 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.

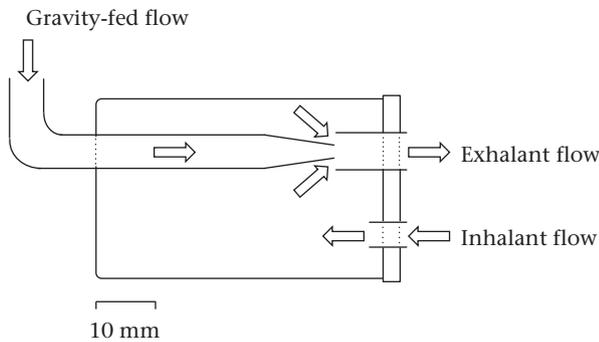


Figure 1. Cross-sectional assembly of artificial mussels used in the study. Arrows indicate direction of water flow.

The bitterling were exposed to all combinations of three experimental mussel treatments, i.e. 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, 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). The mean \pm 95% confidence interval (CI) dissolved oxygen concentration in the high oxygen treatment was 7.48 ± 0.21 mg O₂/litre and in the low oxygen treatment it was 1.48 ± 0.14 mg O₂/litre (Table 2). Algal growth in the 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 the experimental tubs than in the water emerging from artificial mussel siphons, even in the high oxygen treatment (mean \pm 95% CI: high oxygen treatment: 10.24 ± 0.28 mg O₂/litre; low oxygen treatment: 9.87 ± 0.25 mg O₂/litre). Under natural conditions mussels consume between 7% and 90% (Smith et al., 2001) of oxygen flowing over their gills, depending on species, gravity and parasitism by bitterling (Reichard, Liu, & Smith, 2007; Smith et al., 2001, 2000). 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

Table 1
Experimental artificial mussel treatment combinations used in the study

Mussel odour	Dissolved oxygen	Flow velocity
Present	High	High
	Low	Low
Absent	High	High
		Low
	Low	High
		Low

Table 2

Experimental artificial treatments to which European bitterling were exposed in trials

Cue	Treatment	Quantity
Flow velocity	High	300 ml/min
	Low	100 ml/min
Dissolved oxygen	High	7.5 mg/litre
	Low	1.5 mg/litre
Mussel odour	Present	80 mussels/m ³
	Absent	0 mussels/m ³

covariate (see below). The mean \pm 95% CI difference in dissolved oxygen concentration in the high oxygen treatment was 2.77 ± 0.26 mg O₂/litre and in the low oxygen treatment it was 8.40 ± 0.24 mg O₂/litre. The volume of water flowing into the experimental tubs from the artificial mussels during observations (a maximum of 3 litres) was too low to have a measurable impact on oxygen conditions inside the experimental tubs (containing 1000 litres).

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 litres of water and mussels were fed daily with phytoplankton. As mussels filter water at a rate of about 2 litres/h (Smith et al., 2001), 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 2 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 the experimental tubs when fish were moved (approximately 1 litre) would be rapidly diluted.

After the live mussel was replaced with an artificial mussel, the behaviour of the female and focal male was observed for 10 min or until a spawning occurred. For males, we recorded inspection of the exhalant aperture and ejaculation over the inhalant aperture (see Wiepkema, 1961 for full description). For females, we recorded 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 ensuring fertilization of eggs (Smith & Reichard, 2005). Skimming has been proposed as a proxy for female mussel preference (Candolin & Reynolds, 2001; Wiepkema, 1961). 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. Eight artificial mussels were used in the study. Individual artificial mussels were randomized among treatments.

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 variance 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), although with responses distributed equally among treatment combinations. Consequently, zero-altered (hurdle) models with Poisson (ZAP) or negative binomial (ZANB) distributions were employed (Zuur, Ieno, Walker, Saveliev, & Smith, 2009) using the `pscl` package version 1.4.6 (Jackman, 2014) in the R statistical environment, version 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:

$$ejac_i \sim ZANB(\mu_i, \pi_i, k)$$

$$E(ejac_i) = \frac{1 - \pi_i}{1 - P_0} \times \mu_i \quad \text{where } P_0 = \left(\frac{k}{\mu_i + k} \right)^k$$

$$\text{var}(ejac_i) = \frac{1 - \pi_i}{1 - P_0} \times \left(\mu_i + \mu_i^2 + \frac{\mu_i^2}{k} \right) - \left(\frac{1 - \pi_i}{1 - P_0} \times \mu_i \right)^2$$

$$\log(\mu_i) = \text{flow}_i + \text{muss}_i + \text{oxy}_i + \text{oxydiff}_i + \text{msl}_i + \text{fsl}_i + \text{temp}_i + \text{tub}_i + \text{skim}_i$$

$$\text{logit}(\pi_i) = \text{flow}_i + \text{muss}_i + \text{oxy}_i + \text{oxydiff}_i + \text{msl}_i + \text{fsl}_i + \text{temp}_i + \text{tub}_i + \text{skim}_i$$

where $ejac_i$ is the number of ejaculations by focal males in observation i assuming a negative binomial distribution with mean μ , probability π 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 the water temperature of the experimental tub ($^{\circ}\text{C}$), 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:

$$\text{skim}_i \sim ZAP(\mu_i, \pi_i)$$

$$E(\text{skim}_i) = \frac{1 - \pi_i}{1 - e^{-\mu_i}} \times \mu_i$$

$$\text{var}(\text{skim}_i) = \frac{1 - \pi_i}{1 - e^{-\mu_i}} \times (\mu_i + \mu_i^2) - \left(\frac{1 - \pi_i}{1 - e^{-\mu_i}} \times \mu_i \right)^2$$

$$\log(\mu_i) = \text{flow}_i + \text{muss}_i + \text{oxy}_i + \text{oxydiff}_i + \text{msl}_i + \text{fsl}_i + \text{temp}_i + \text{tub}_i + \text{ejac}_i$$

$$\text{logit}(\pi_i) = \text{flow}_i + \text{muss}_i + \text{oxy}_i + \text{oxydiff}_i + \text{msl}_i + \text{fsl}_i + \text{temp}_i + \text{tub}_i + \text{ejac}_i$$

where skim_i is the number of skims by focal females in observation i assuming a Poisson distribution with mean μ and probability π (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 version 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 data sets from the best-fitting models and compared these with observed data, using the procedure of Zuur and Ieno (2016) for hurdle models.

Ethical Note

The experimental protocol was noninvasive, 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 libitum 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. Electro-fishing was considered the least stressful method of capture (Janáč, 2009), with much lower impacts on nontarget 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 (Fig. 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 (Fig. 2, Table 3). Similarly, both mussel odour cues and a high dissolved oxygen concentration were needed for eliciting female skimming behaviour over artificial mussels (Fig. 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 the experimental tubs and that emerging from the artificial mussel siphon (Fig. 3, Table 3). There was also a negative association between female skimming frequency and focal male size (Fig. 3,

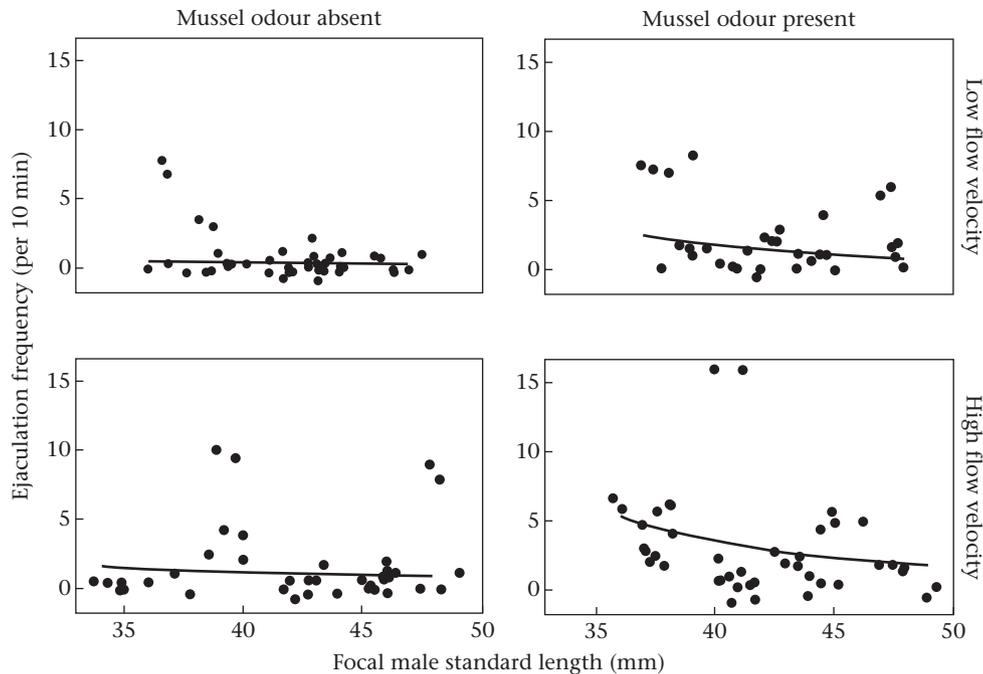


Figure 2. Ejaculation frequency (per 10 min) by focal male *R. amarus* over an artificial mussel with and without mussel odour cues and low and high flow velocity against focal male standard length (mm) modelled using a zero-altered negative binomial (ZANB) model. Black circles are observed data.

Table 3

Results of best-fit zero-altered negative binomial (ZANB, males) and zero-altered Poisson (ZAP, females) models testing the effects of mussel parameters on the responses of *R. amarus*

Sex	Parameter	Occurrence model				Frequency model			
		γ	SE	Z	P	β	SE	Z	P
Male	Intercept	-1.34	2.99	-0.45	0.654	5.77	2.16	2.68	0.008
	Flow _(high)	—	—	—	—	0.83	0.42	1.99	0.047
	Mussel _(present)	1.99	0.52	3.84	<0.001	—	—	—	—
	DO _(high)	0.91	0.52	1.76	0.079	0.24	0.42	0.56	0.577
	Male size	-0.01	0.07	-0.04	0.971	-0.11	0.05	-2.15	0.031
	Skimming	—	—	—	—	-0.15	0.11	-1.32	0.187
Female	Intercept	-1.95	3.94	-0.50	0.620	-2.97	60.03	-0.05	0.961
	Flow _(high)	—	—	—	—	-0.14	0.35	-0.40	0.688
	Mussel _(present)	3.28	1.09	3.02	0.003	9.89	60.00	0.16	0.869
	DO _(high)	1.50	0.70	2.15	0.032	—	—	—	—
	O ₂ difference	—	—	—	—	0.19	0.07	2.62	0.009
	Male size	-0.06	0.09	-0.70	0.481	-0.17	0.06	-2.57	0.010

Bold text indicates $P < 0.05$.

Table 3). Simulated data generated from our best-fit models generated distributions that were concordant 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, we determined its magnitude by fitting zero-altered statistical models (Hilbe, 2014; Zuur et al., 2009). Both sexes responded positively 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 (Kitamura, Nagata, Nakajima, & Sota, 2012; Liu, Yurong, Reichard, & Smith, 2006). In these cases, species-specific odour cues may play a role in mussel choice (Reichard, Liu et al., 2007) 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, Gibernau, & Frey, 1994), mosquitos (Afify & Galizia, 2015) and parasitoids (Godfray, 1994). In *Drosophila 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.

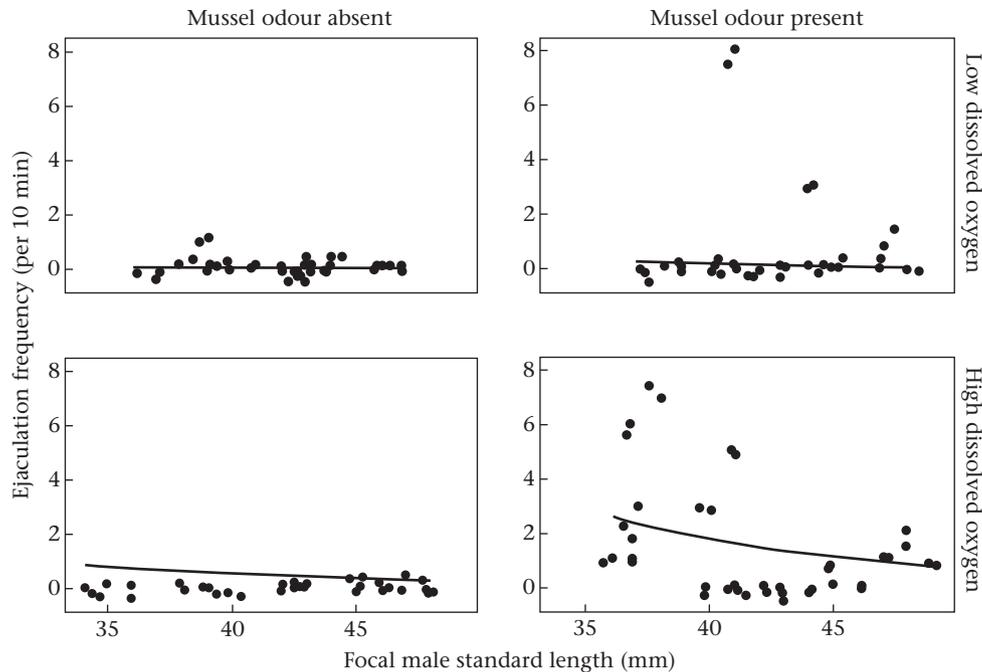


Figure 3. Skimming frequency (per 10 min) by focal females over an artificial mussel with and without mussel odour cues and low and high dissolved oxygen concentrations in the exhalant flow against focal male standard length (mm) modelled using a zero-altered Poisson (ZAP) model. Black circles are observed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

Bitterling eggs are relatively large compared to those of 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 (Kitamura, 2005; Smith et al., 2001), competition for oxygen inside the mussel gill can be severe, both between embryos and between embryos and host, and it is notable that embryo mortality rates in mussels are strongly density dependent (Agbali & Smith, 2012; Smith et al., 2001, 2000; 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 caused by 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 (Gandon, Rivero, & Varaldi, 2006; Godfray, 1994) 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 (Harvey, 2000; van der Hoeven & Hemerik, 1990; Viser, 1993). 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 exhalant flow may operate in the bitterling system.

While female *R. amarus* showed a significant threshold response to high dissolved oxygen conditions, the strength of the 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 several 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, Smith, & Campbell, 2002). In salmonids, females assess substrate quality and hyporheic flow prior to preparing spawning redds and oviposition (Bernier-Bourgault & Magnan, 2002; Brabrand, Koestler, & Borgström, 2002; Chapman, 1988; 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: they did not ejaculate exclusively in high-flow mussels, but adjusted 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, Pateman-Jones, Zięba, Przybylski, & Reichard, 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, Warren, Rouchet, & Reichard, 2014). Filtration rates vary naturally between (Smith et al., 2001) and within host mussel species (Mills & Reynolds, 2002b). Smith and 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 (Fig. 3). Thus, while variation in mussel flow rates did not inhibit male host preference, our results demonstrate that males can adjust their behaviour adaptively to their current host.

Male size was negatively associated with the frequency of ejaculation and the female response to mussels (Table 3). Male size determines dominance in bitterling (Casalini et al., 2009; Smith, Reichard, & Jurajda, 2003), 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 (although not absolutely) larger testes (Smith, Warren et al., 2014) and typically compete with rivals through sperm competition rather than direct aggressive contests (Reichard, Smith, & Jordan, 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, although large dominant males are typically able to monopolize mussels and thereby to achieve high reproductive success (Casalini et al., 2009; Reichard, LeComber, Smith, 2007; Reichard, Ondračková, Bryjová, Smith, & Bryja, 2009). Male nuptial colour similarly has not been demonstrated to have a direct effect on female mate choice (Casalini et al., 2009; Reichard et al., 2005). Without measuring further male traits such as genetic compatibility, we are unable to account for this apparent elevated response by females to smaller males.

Conclusion

Overall our results demonstrate 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 the surrounding water. 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 incubating young stages (Agbali, Reichard, Bryjová, Bryja, & Smith, 2010; Agbali & Smith, 2012; Smith, Douglas, & Jurajda, 2002, 2001), males instead appear sensitive to the risk of sperm competition (Spence, Reichard, & Smith, 2013), and are insensitive to mussel quality (Casalini et al., 2013; Smith, Phillips, Polacik, Reichard, 2014; Smith et al., 2003, 2002). Consequently, a sexual conflict over responses to oviposition sites in *R. amarus* may 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 by 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 by their suitability for their offspring. We are aware of no other mating system with conflicting responses to oviposition site cues like that seen in *R. amarus*.

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References

- Adolph, E. F. (1920). Egg-laying reactions in the pomace fly, *Drosophila*. *Journal of Experimental Zoology*, 31, 326–341.
- Afify, A., & Galizia, C. G. (2015). Chemosensory cues for mosquito oviposition site selection. *Journal of Medical Entomology*, 52, 120–130.
- Agbali, M., Reichard, M., Bryjová, A., Bryja, J., & Smith, C. (2010). Mate choice for nonadditive genetic benefits correlate with MHC dissimilarity in the rose bitterling (*Rhodeus ocellatus*). *Evolution*, 64, 1683–1696.
- Agbali, M., & Smith, C. (2012). Long-term fitness consequences for offspring of female oviposition decisions in the rose bitterling (*Rhodeus ocellatus*). *Acta Zoologica*, 93, 367–372.
- Akaike, H. (1973). Maximum likelihood identification of Gaussian autoregressive moving average models. *Biometrika*, 60, 255–265.
- Aldridge, D. C. (1999). Development of European bitterling in the gills of freshwater mussels. *Journal of Fish Biology*, 54, 138–151.
- Arnqvist, G., & Rowe, L. (2005). *Sexual conflict*. Princeton, NJ: Princeton University Press.
- Bernier-Bourgault, I., & Magnan, P. (2002). Factors affecting redd site selection, hatching, and emergence of brook charr, *Salvelinus fontinalis*, in an artificially enhanced site. *Environmental Biology of Fishes*, 64, 333–341.
- Brabrand, Å., Koestler, A. G., & Borgström, R. (2002). Lake spawning of brown trout related to groundwater influx. *Journal of Fish Biology*, 60, 751–763.
- Candolin, U., & Reynolds, J. D. (2001). Sexual signaling in the European bitterling: Females learn the truth by direct inspection of the resource. *Behavioral Ecology*, 12(4), 407–411.
- Candolin, U., & Reynolds, J. D. (2002). Adjustments of ejaculation rates in response to risk of sperm competition in a fish, the bitterling (*Rhodeus sericeus*). *Proceedings of the Royal Society B: Biological Sciences*, 269(1500), 1549–1553.
- Casalini, M., Agbali, M., Reichard, M., Konečná, M., Bryjová, A., & Smith, C. (2009). Male dominance, female mate choice, and intersexual conflict in the rose bitterling (*Rhodeus ocellatus*). *Evolution*, 63, 366–376.
- Casalini, M., Reichard, M., Phillips, A., & Smith, C. (2013). Male choice of mates and mating resources in the rose bitterling (*Rhodeus ocellatus*). *Behavioural Ecology*, 24, 1199–1204.
- Chapman, D. W. (1988). Critical review of variables used to define effects of fines in redds of large salmonids. *Transactions of the American Fisheries Society*, 117, 1–21.
- Clutton-Brock, T. H. (1991). *The evolution of parental care*. Princeton, NJ: Princeton University Press.
- Cope, J. M., & Fox, C. W. (2003). Oviposition decisions in the seed beetle, *Callosobruchus maculatus* (Coleoptera: Bruchidae): Effects of seed size on superparasitism. *Journal of Stored Products Research*, 39, 355–365.
- Córdoba-Aguilar, A. (2009). A female evolutionary response when survival is at risk: Male harassment mediates early reallocation of resources to increase egg number and size. *Behavioural Ecology and Sociobiology*, 63, 751–763.
- Davenport, J., & Woolmington, A. D. (1982). A new method of monitoring ventilatory activity in mussels and its use in a study of the ventilatory patterns of *Mytilus edulis* L. *Journal of Experimental Marine Biology and Ecology*, 62, 55–67.

- Dweck, H. K., Ebrahim, S. A., Kromann, S., Bown, D., Hillbur, Y., Sachse, S., et al. (2013). Olfactory preference for egg laying on citrus substrates in *Drosophila*. *Current Biology*, 23, 2472–2480.
- Esteve, M. (2005). Observations of spawning behaviour in Salmoninae: *Salmo*, *Oncorhynchus* and *Salvelinus*. *Reviews of Fish Biology and Fisheries*, 15, 1–21.
- Gandon, S., Rivero, A., & Valaldi, J. (2006). Superparasitism evolution: Adaptation or manipulation? *American Naturalist*, 167, E1–E22.
- Godfray, H. C. J. (1994). *Parasitoids*. Princeton, NJ: Princeton University Press.
- Harvey, J. A. (2000). Dynamic effects of parasitism by an endoparasitoid wasp on the development of two host species: Implications for host quality and parasitoid fitness. *Ecological Entomology*, 25, 267–278.
- Heschl, A. (1989). Integration of “innate” and “learned” components within the IRME for mussel recognition in the European bitterling *Rhodeus amarus* (Bloch). *Ethology*, 81, 193–208.
- Hilbe, J. M. (2014). *Modeling count data*. Cambridge, U.K.: Cambridge University Press.
- van der Hoeven, N., & Hemerik, L. (1990). Superparasitism as an ESS: To reject or not to reject, that is the question. *Journal of Theoretical Biology*, 146, 467–482.
- Hossaert-McKey, M., Gibernau, M., & Frey, J. E. (1994). Chemosensory attraction of fig wasps to substances produced by receptive figs. *Entomologia Experimentalis et Applicata*, 70, 185–191.
- Ieno, E. N., & Zuur, A. F. (2015). *Data exploration and visualisation with R*. Newburgh, U.K.: Highland Statistics Ltd.
- Jackman, S. (2014). *pscl: Classes and methods for R developed in the political science computational laboratory, Stanford University (R package version 1.4.6)*. Stanford, CA: Stanford University.
- Janáč, M. (2009). *Sampling and evaluation of the young-of-the-year fish assemblages* (Doctoral dissertation). Brno, Czech Republic: Masarykova Univerzita.
- Kitamura, J. (2005). Factors affecting seasonal mortality of rosy bitterling (*Rhodeus ocellatus kurumeus*) embryos on the gills of their host mussel. *Population Ecology*, 47, 41–51.
- Kitamura, J. (2006). Adaptive spatial utilization of host mussels by the Japanese rosy bitterling *Rhodeus ocellatus kurumeus*. *Journal of Fish Biology*, 69, 263–271.
- Kitamura, J., Nagata, N., Nakajima, J., & Sota, T. (2012). Divergence of ovipositor length and egg shape in a brood parasitic bitterling fish through the use of different mussel hosts. *Journal of Evolutionary Biology*, 25, 566–573.
- Konečná, M., & Reichard, M. (2011). Seasonal dynamics in population characteristics of European bitterling *Rhodeus amarus* in a small lowland river. *Journal of Fish Biology*, 78, 227–239.
- Lessells, C. M. (2006). The evolutionary outcome of sexual conflict. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 361, 301–317.
- Liu, H., Yurong, Z., Reichard, M., & Smith, C. (2006). Evidence of host specificity and congruence between phylogenies of bitterlings and freshwater mussels. *Zoological Studies*, 45, 428–434.
- Mayhew, P. J. (1997). Adaptive patterns of host-plant selection by phytophagous insects. *Oikos*, 79, 417–428.
- Mazerolle, M. J. (2016). *AICcmovavg: model selection and multimodel inference based on (Q)AIC(c)*. R package version 2.1.0. Available at: <http://CRAN.R-project.org>.
- Mills, S. C., & Reynolds, D. C. (2002a). Host species preferences by bitterling, *Rhodeus sericeus*, spawning in freshwater mussels and consequences for offspring survival. *Animal Behaviour*, 63, 1029–1036.
- Mills, S. C., & Reynolds, D. C. (2002b). Mussel ventilation rates as a proximate cue for host selection by bitterling, *Rhodeus sericeus*. *Oecologia*, 131, 473–478.
- Mousseau, T. A., & Fox, C. W. (1998). The adaptive significance of maternal effects. *Trends in Ecology and Evolution*, 13, 403–407.
- Parker, G. A. (1979). Sexual selection and sexual conflict. In M. S. Blum, & N. A. Blum (Eds.), *Sexual selection and reproductive competition in insects* (pp. 123–166). New York, NY: Academic Press.
- Parker, G. A. (1998). Sperm competition and the evolution of ejaculates: Towards a theory base. In T. R. Birkhead, & A. P. Møller (Eds.), *Sperm competition and sexual selection* (pp. 3–54). London, U.K.: Academic Press.
- Payne, A. G., Smith, C., & Campbell, A. C. (2002). Filial cannibalism improves survival and development of beaugregory damselfish embryos. *Proceedings of the Royal Society B: Biological Sciences*, 269, 2095–2102.
- Qvarnström, A., & Forsgren, E. (1998). Should females prefer dominant males? *Trends in Ecology and Evolution*, 13, 498–501.
- R Development Core Team. (2016). *R: A language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing. Available at: <http://www.R-project.org/>.
- Refsnider, J. M., & Janzen, F. J. (2010). Putting eggs in one basket: Ecological and evolutionary hypotheses for variation in oviposition-site choice. *Annual Reviews of Ecology, Evolution and Systematics*, 41, 39–57.
- Reichard, M., Bryja, J., Ondračková, M., Dávidová, M., Kaniewska, P., & Smith, C. (2005). Sexual selection for male dominance reduces opportunities for female mate choice in the European bitterling (*Rhodeus sericeus*). *Molecular Ecology*, 14(5), 1533–1542.
- Reichard, M., LeComber, S. C., & Smith, C. (2007). Sneaking from a female perspective. *Animal Behaviour*, 74, 679–688.
- Reichard, M., Liu, H., & Smith, C. (2007). The co-evolutionary relationship between bitterling fishes and freshwater mussels: Insights from interspecific comparisons. *Evolutionary Ecology Research*, 9, 239–259.
- Reichard, M., Ondračková, M., Bryjová, A., Smith, C., & Bryja, J. (2009). Breeding resource distribution affects selection gradients on male phenotypic traits: Experimental study on lifetime reproductive success in the bitterling fish (*Rhodeus amarus*). *Evolution*, 63(2), 377–390.
- Reichard, M., Ondračková, M., Przybylski, M., Liu, H., & Smith, C. (2006). The costs and benefits in an unusual symbiosis: Experimental evidence that bitterling fish (*Rhodeus sericeus*) are parasites of unionid mussels in Europe. *Journal of Evolutionary Biology*, 19, 788–796.
- Reichard, M., Smith, C., & Jordan, W. C. (2004). Genetic evidence reveals density-dependent mediated success of alternative mating behaviours in the European bitterling (*Rhodeus sericeus*). *Molecular Ecology*, 13, 1569–1578.
- Riffell, J. A. (2013). Neuroethology: Lemon-fresh scent makes flies lay eggs. *Current Biology*, 23, R1108–R1110.
- Roitberg, B. D. (1998). Oviposition decisions as maternal effects: Conundrums and opportunities for conservation biologists. In T. A. Mousseau, & C. W. Fox (Eds.), *Maternal effects as adaptations* (pp. 67–81). Oxford, U.K.: Oxford University Press.
- Shine, R., & Harlow, P. S. (1996). Maternal manipulation of offspring phenotypes via nest-site selection in an oviparous lizard. *Ecology*, 77, 1808–1817.
- Smith, C. (2017). Bayesian inference supports the host selection hypothesis in explaining adaptive host specificity by European bitterling. *Oecologia*, 183, 379–389.
- Smith, C., Douglas, A., & Jurajda, P. (2002). Sexual conflict, sexual selection and sperm competition in the spawning decisions of bitterling, *Rhodeus sericeus*. *Behavioural Ecology and Sociobiology*, 51, 433–439.
- Smith, C., Pateman-Jones, C., Zięba, G., Przybylski, M., & Reichard, M. (2009). Sperm depletion as a consequence of increased sperm competition risk in the European bitterling, *Rhodeus amarus*. *Animal Behaviour*, 77, 1227–1233.
- Smith, C., Phillips, A., Poláčik, M., & Reichard, M. (2014). Male coloration signals direct benefits in the European bitterling (*Rhodeus amarus*). *Environmental Biology of Fishes*, 97, 335–341.
- Smith, C., Phillips, A., & Reichard, M. (2015). Cognitive ability is heritable and predicts the success of an alternative mating tactic. *Proceedings of the Royal Society B: Biological Sciences*, 282, 20151046.
- Smith, C., & Reichard, M. (2005). Females solicit sneakers to improve fertilization success in the bitterling fish (*Rhodeus sericeus*). *Proceedings of the Royal Society B: Biological Sciences*, 272, 1683–1688.
- Smith, C., & Reichard, M. (2013). A sperm competition model for the European bitterling (*Rhodeus amarus*). *Behaviour*, 150, 1709–1730.
- Smith, C., Reichard, M., & Jurajda, P. (2003). Assessment of sperm competition by European bitterling, *Rhodeus sericeus*. *Behavioural Ecology and Sociobiology*, 53, 206–213.
- Smith, C., Reichard, M., Jurajda, P., & Przybylski, M. (2004). The reproductive ecology of the European bitterling (*Rhodeus sericeus*). *Journal of Zoology*, 262, 107–124.
- Smith, C., Reynolds, J. D., Sutherland, W. J., & Jurajda, P. (2000). Adaptive host choice and avoidance of superparasitism in the spawning decisions of bitterling (*Rhodeus sericeus*). *Behavioural Ecology and Sociobiology*, 48, 29–35.
- Smith, C., Rippon, K., Douglas, A., & Jurajda, P. (2001). A proximate cue for oviposition site choice in the bitterling (*Rhodeus sericeus*). *Freshwater Biology*, 46, 903–911.
- Smith, C., Warren, M., Rouchet, R., & Reichard, M. (2014). The function of multiple ejaculations in bitterling. *Journal of Evolutionary Biology*, 27, 1819–1829.
- Smith, G., & Ritchie, M. G. (2013). How might epigenetics contribute to ecological speciation? *Current Zoology*, 59, 686–696.
- Soler, M. (2014). Long-term coevolution between avian brood parasites and their hosts. *Biological Reviews*, 89, 688–704.
- Spence, R., Reichard, M., & Smith, C. (2013). Strategic sperm allocation and a Coolidge effect in an externally fertilizing species. *Behavioural Ecology*, 24, 82–88.
- Spence, R., & Smith, C. (2005). Male territoriality mediates density and sex ratio effects on oviposition in the zebrafish, *Danio rerio*. *Animal Behaviour*, 69, 1317–1323.
- Spence, R., & Smith, C. (2013). Rose bitterling (*Rhodeus ocellatus*) embryos parasitize freshwater mussels by competing for nutrients and oxygen. *Acta Zoologica*, 94, 113–118.
- Stadnichenko, A. P., & Stadnichenko, Y. A. (1980). On the effect of bitterling larvae on the lamellibranchid mollusc *Unio rostratus gentilis* Haas. *Gidrobiologicheskii Zhurnal*, 1980, 57–61.
- Taylor, A. J., Müller, C. B., & Godfray, H. C. J. (1998). Effect of aphid predators on oviposition behavior of aphid parasitoids. *Journal of Insect Behaviour*, 11, 297–302.
- Tinbergen, N. (1951). *The study of instinct*. Oxford, U.K.: Oxford University Press.
- Viser, M. E. (1993). Adaptive self-and conspecific superparasitism in the solitary parasitoid *Leptopilina heterotoma* (Hymenoptera: Eucoilidae). *Behavioural Ecology*, 4, 22–28.
- Wiepkema, P. R. (1961). An ethological analysis of the reproductive behaviour of the bitterling (*Rhodeus amarus* Bloch). *Archives Néerlandaises de Zoologie*, 14, 103–199.
- Wolfner, M. F. (2002). The gifts that keep on giving: Physiological functions and evolutionary dynamics of male seminal proteins in *Drosophila*. *Heredity*, 88, 85–93.
- Wootton, R. J., & Smith, C. (2015). *Reproductive biology of teleost fishes*. Oxford, U.K.: Wiley-Blackwell.
- Zuur, A. F., & Ieno, E. N. (2016). A protocol for conducting and presenting results of regression-type analyses. *Methods in Ecology and Evolution*, 7(6), 636–645.
- Zuur, A., Ieno, E. N., Walker, N., Saveliev, A. A., & Smith, G. M. (2009). *Mixed effects models and extensions in ecology with R*. New York, NY: Springer.