

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

3 ABSTRACT

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

13 Keywords

14 maternal effect, mussel, oviposition-site cue, *Rhodeus*, reproduction, sexual conflict

15 INTRODUCTION

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

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

34 The oviposition-site decisions of males and females need not correspond. In some mating
35 systems males can influence female oviposition-site decisions through harassment (Córdoba-
36 Aguilar, 2009), the transfer of ejaculatory substances during mating (Wolfner, 2002), and by
37 controlling access to oviposition sites (Qvarnström & Forsgren, 1998), potentially resulting in
38 sexual conflict (Spence & Smith, 2005). If oviposition-site decisions have different outcomes for
39 the sexes and represent a possible arena for sexual conflict, a key question is whether the sexes
40 attend to the same or different cues in making decisions.

41 Here we investigate the responses of male and female European bitterling (*Rhodeus amarus*)
42 to oviposition-site cues. *R. amarus* are a small freshwater cyprinid fish that use living unionid
43 mussels for oviposition (Smith et al., 2004). Males defend small territories focused on one or
44 more freshwater mussels (Tinbergen, 1951; Wiepkema, 1961), to which they 'lead' females to
45 spawn a clutch of 1-6 eggs. Females use a long ovipositor to place their eggs inside the gill cavity
46 of a mussel and the male fertilizes the eggs by releasing sperm over the mussel (Smith et al.,
47 2004). Bitterling embryos complete development inside the mussel gill, imposing significant
48 costs on the host for their month-long development. Females lay approximately 250 eggs over the
49 course of a single spawning season and, therefore, make multiple oviposition-site decisions
50 (Smith et al., 2004). Spawning in bitterling often involves 'sneaky' matings, whereby a male that
51 has not courted a female may successfully participate in spawning (Smith et al., 2015).
52 Developing bitterling eggs and embryos compete with the host for oxygen and nutrients (Spence
53 & Smith, 2013), limiting mussel growth and fecundity (Reichard et al., 2006) and potentially
54 damaging gill tissue (Stadnichenko & Stadnichenko, 1980). Multiple clutches can be deposited in
55 the same mussel, and egg and embryo mortality is strongly density dependent (Smith et al., 2000;
56 Spence & Smith, 2013). Different mussel species vary in quality as hosts, and bitterling are
57 choosy about which mussels are used for oviposition (Smith et al., 2000; Casalini et al., 2013).
58 Thus oviposition-site decisions are a key component of the bitterling mating system, with
59 immediate (Smith et al., 2000; Mills & Reynolds, 2002a) and longer-term (Agbali & Smith,
60 2012) consequences for fitness.

61 The cues used by bitterling for oviposition-site decisions are ambiguous. *R. amarus* show a
62 response to water flow from the exhalant siphon of a mussel (Smith et al., 2001; Mills &
63 Reynolds, 2002b), but also to the dissolved oxygen concentration of the exhalant flow (Smith et
64 al., 2000, 2001). Separating the effects of these two cues is problematic because flow velocity
65 and dissolved oxygen concentration are potentially correlated (Davenport & Woolmington,
66 1982). Additionally, there is evidence that visual and odour cues and the presence and behaviour

67 of other bitterling can influence oviposition choice (Heschl, 1989; Smith & Reichard, 2005).
68 Males and females may not express the same host preferences. Casalini et al. (2013) suggested
69 that males tracked female host preferences and it may be the case that male behaviour does not
70 directly indicate preference for a mussel, but instead represents adaptive plastic behaviour
71 towards a host. Here we experimentally investigated the strength of response of *R. amarus* to
72 oviposition site cues. We examined three discrete cues; water flow velocity, dissolved oxygen
73 concentration, and mussel odour, using an artificial mussel that permitted us to manipulate each
74 cue independently. We addressed the question of whether single or multiple cues are used and
75 whether males and females use the same or different cues.

76

77 METHODS

78 *General methods*

79 Approximately 350 *R. amarus* were collected from a river at the centre of the distribution of the
80 fish in Europe. In addition, approximately 180 *Unio tumidus* mussels were collected from an
81 adjacent oxbow lake (where both bitterling and mussels are abundant) prior to the start of the
82 spawning season during April 2015. Fish and mussels were transported to outdoor fiberglass tubs
83 (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
84 dechlorinate for three days and furnished with a gravel substrate and artificial plants as refuges.
85 Fish were stored in mixed sex groups at low densities (approximately 30 fish per tub) and fed *ad*
86 *libitum* three times daily with a mixture of frozen chironomid larvae and copepods. Mussels were
87 stored separately from fish. Approximately one third of the water in tubs containing fish and
88 mussels was changed twice weekly to maintain water quality. Given the low densities of
89 bitterling, which are small fish (typically < 60 mm standard length), poor water quality was not a
90 problem during the study. Fish and mussels in tubs were exposed to natural light and temperature
91 variation, typical for mid-May in central Europe. Mean (\pm SD) water temperature was 17.9 (\pm

92 2.5) °C, and there were approximately 15.5 h daylight hours each day over the experimental
93 period.

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

105 To start the experiment a female with an extended ovipositor, indicating a readiness to spawn,
106 was gently caught in one of the stock tubs and transferred to a glass box measuring 220 (height) x
107 80 (width) x 80 (depth) mm with a mesh top to permit water exchange. The female was placed in
108 a pre-selected experimental tub 300 mm from the mussel guarded by the focal male. Once the
109 focal male began courtship and the female showed a response by attempting to follow him, the
110 live *U. tumidus* was replaced with an artificial mussel and the female was released from the glass
111 box. Any odour from the live mussel was diluted quickly within the 1000 litre experimental tub
112 and so would not have affected behaviour towards the artificial mussel. This experimental design
113 was intended to accommodate the mating system of European bitterling. In nature, males are
114 highly territorial around a patch of mussels, while females display no site attachment and range
115 among male territories, feeding and spawning over an extensive area (Smith et al., 2004). Thus
116 the design we used, with males confined to a territory, and gravid females gently introduced to
117 these territories for short intervals, mirrored natural conditions.

118 Artificial mussels comprised a 35 mm plastic film canister measuring 50 (length) x 30
119 (diameter) mm with a snap on lid. The lid of the canister had two openings; an exhalant aperture
120 of 10 mm and an inhalant aperture of 5 mm. The female bitterling releases eggs through the
121 exhalant siphon while the male releases sperm over the inhalant siphon. A Venturi system
122 generated an exhalant and inhalant flow. Water flowed into the base of the artificial mussel under
123 gravity, through a constriction to elevate flow velocity, and out of the exhalant aperture (Figure
124 1). The elevated water flow velocity and reduced static pressure generated by the constriction
125 created an inward flow of water through the inhalant aperture of the artificial mussel (Figure 1).
126 This design of artificial mussel permitted the source and rate of flow to be experimentally
127 manipulated. In pilot studies males guarded artificial mussels, led females to them to spawn and
128 ejaculated over the inhalant aperture. Similarly, females inspected the exhalant aperture of
129 artificial mussels and spawned in them. We detected no negative effects of potential endocrine-
130 disrupting chemicals derived from artificial mussels. Even if present, the dilution of these
131 chemicals in experimental tubs, combined with the extremely short time to which fish were
132 exposed to them, meant that the reproductive system of experimental fish was unlikely to have
133 been compromised.

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

139 Artificial mussels were connected by 5 mm diameter PVC tubing to a 1000 litre reservoir
140 ('source tub') that was raised approximately 0.6 m above the level of the experimental tub in
141 which observations were carried out. To create a high dissolved oxygen concentration (DO),
142 water in the reservoir was strongly aerated with an air pump. To create a low dissolved oxygen
143 concentration, nitrogen was bubbled through water in the reservoir. Dissolved oxygen

144 concentration was monitored with a dissolved oxygen meter (HORIBA U-222). Mean \pm 95% CI
145 dissolved oxygen concentration in high oxygen treatment reservoirs was 7.48 ± 0.21 mg O₂ /litre,
146 and low treatment 1.48 ± 0.14 mg O₂ /litre (Table 1). Algal growth in experimental tubs resulted
147 in elevated dissolved oxygen concentrations through photosynthesis compared to source tubs,
148 which were free of algae. The outcome was a higher ambient dissolved oxygen level in
149 experimental tubs than in the water emerging from artificial mussel siphons, even in the high
150 dissolved oxygen treatment (mean \pm 95% CI high dissolved oxygen treatment 10.24 ± 0.28 mg
151 O₂ /litre, low treatment 9.87 ± 0.25 mg O₂ /litre). Under natural conditions mussels consume
152 between 7% and 90% (Smith et al. 2001) of oxygen flowing over their gills, depending on
153 species, gravidity and parasitism by bitterling (Smith et al., 2000, 2001, Reichard et al., 2007a).
154 Therefore, the dissolved oxygen concentration of water emerging from the siphons of artificial
155 mussels, which declined to between 73% and 15% of the concentration of the surrounding water,
156 accurately reflected the range naturally encountered by bitterling. To accommodate this feature of
157 the study in our analysis, the difference in the ambient dissolved oxygen concentration in
158 experimental tubs and the source tub supplying water to the artificial mussel was calculated and
159 used as an additional covariate (see below). The mean \pm 95% CI difference in dissolved oxygen
160 concentration in the high oxygen treatment was 2.77 ± 0.26 mg O₂ /litre, and low treatment 8.40
161 ± 0.24 mg O₂ /litre. The volume of water flowing into the experimental tubs from the artificial
162 mussels during observations (a maximum of 3 litre) was too low to have a measurable impact on
163 oxygen conditions inside the experimental tubs (containing 1000 litre).

164 High and low flow rates were obtained by clamping the tube connecting the artificial mussel to
165 the water source until the desired flow rate was achieved. The presence of mussel odour was
166 achieved by placing 80 *U. tumidus* mussels in the 1000 litre source reservoir, a procedure
167 previously used to elicit oviposition behaviour in *R. amarus* (Heschl 1989). Water quality was
168 maintained with twice weekly water changes of approximately 250 litre of water and mussels
169 were fed daily with phytoplankton. Mussels filter water at a rate of about 2 litres /hour (Smith et

170 al., 2001), hence the entire contents of the source reservoir would pass across the gills of the
171 stocked mussels several times in 24 h. The source tubs were stocked with mussels two weeks
172 prior to the start of the experiment to ensure a maximum concentration of odour was reached and
173 pilot trials showed that bitterling were responsive to this concentration of odour. Any mussel
174 odour cues in the small quantities of water transferred to experimental tubs when fish were
175 moved (approximately 1 litre) would be rapidly diluted.

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

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

195 *Statistical analysis*

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

209 The data contained a high incidence of zero counts (50% for ejaculation frequency, 80% for
 210 skimming behaviour), though with responses distributed equitably among treatment
 211 combinations. Consequently, zero-altered (hurdle) models with Poisson (ZAP) or negative
 212 binomial (ZANB) distributions were employed (Zuur et al., 2009) using the *pscl* package ver.
 213 1.4.6 (Jackman, 2014) in the R statistical environment, ver. 3.3.2 (R Development Core Team,
 214 2016). Zero-altered models are partitioned into two parts, with a binary process modelling zeros
 215 and positive counts, and a second process modelling only positive counts using a zero-truncated
 216 model (Hilbe, 2014). This modelling approach enabled us to separately identify the mussel cues
 217 that elicited the occurrence of a behaviour (binary part), and the frequency of that behaviour
 218 when it occurred (zero-truncated part). For males a ZANB model was fitted as:

$$219 \quad ejac_i \sim ZANB(\mu_i, \pi_i, k)$$

$$220 \quad E(ejac_i) = \frac{1 - \pi_i}{1 - P_0} \times \mu_i \quad \text{where } P_0 = \left(\frac{k}{\mu_i + k}\right)^k$$

$$221 \quad var(ejac_i) = \frac{1 - \pi_i}{1 - P_0} \times \left(\mu_i + \mu_i^2 + \frac{\mu_i^2}{k}\right) - \left(\frac{1 - \pi_i}{1 - P_0} \times \mu_i\right)^2$$

222
$$\log(\mu_i) = flow_i + muss_i + oxy_i + oxydiff_i + msl_i + fsl_i + temp_i + tub_i + skim_i$$

223
$$\text{logit}(\pi_i) = flow_i + muss_i + oxy_i + oxydiff_i + msl_i + fsl_i + temp_i + tub_i + skim_i$$

224 Where $ejac_i$ is the number of ejaculations by focal males in observation i assuming a negative
 225 binomial distribution with mean μ , probability π and dispersion k (Zuur et al., 2009). The
 226 variables $flow_i$, $muss_i$ and oxy_i are categorical covariates with two levels corresponding with
 227 artificial mussel water flow, mussel odour and dissolved oxygen, respectively. The variables
 228 $oxydiff_i$, msl_i , fsl_i , and $temp_i$ are continuous covariates corresponding with difference in dissolved
 229 oxygen concentration between artificial mussel and experimental tub (mg/litre), male standard
 230 length (mm), female standard length (mm) and water temperature of experimental tub ($^{\circ}\text{C}$),
 231 respectively. The variable tub_i was included to control for an effect of experimental tub and $skim_i$
 232 was a continuous covariate that corresponded with female skimming frequency and was included
 233 to accommodate the effect of female behaviour on male mussel preferences.

234 For females a ZAP model was fitted as:

235
$$skim_i \sim ZAP(\mu_i, \pi_i)$$

236
$$E(skim_i) = \frac{1 - \pi_i}{1 - e^{-\mu_i}} \times \mu_i$$

237
$$\text{var}(skim_i) = \frac{1 - \pi_i}{1 - e^{-\mu_i}} \times (\mu_i + \mu_i^2) - \left(\frac{1 - \pi_i}{1 - e^{-\mu_i}} \times \mu_i\right)^2$$

238
$$\log(\mu_i) = flow_i + muss_i + oxy_i + oxydiff_i + msl_i + fsl_i + temp_i + tub_i + ejac_i$$

239
$$\text{logit}(\pi_i) = flow_i + muss_i + oxy_i + oxydiff_i + msl_i + fsl_i + temp_i + tub_i + ejac_i$$

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

243 Best-fit zero-altered models were selected based on second-order Akaike's information
 244 criterion (AICc; Akaike, 1973) using the *AICcmodavg* package ver. 2.1-0 (Mazerolle, 2016) by
 245 removing predictor variables from the full models until the model with the lowest AICc values
 246 were identified. To assess the robustness of each model we simulated 1000 datasets from the

247 best-fitting models and compared these with observed data, using the procedure of Zuur & Ieno
248 (2016) for hurdle models.

249 *Ethical Note*

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

259

260 RESULTS

261 The presence of mussel odour cues was essential for determining whether males responded to a
262 mussel with ejaculations (Figure 2, Table 3). In the presence of odour cues, the frequency of
263 ejaculation was positively associated with high water flow velocity but negatively with male size
264 (Figure 2, Table 3). Similarly, the presence of both mussel odour cues and a high dissolved
265 oxygen concentration was needed for eliciting female skimming behaviour over artificial mussels
266 (Figure 3, Table 3). In the presence of these cues, the frequency of skimming by females was
267 positively related to the magnitude of the difference between ambient dissolved oxygen in
268 experimental tubs and that emerging from the artificial mussel siphon (Figure 3, Table 3). There
269 was also a negative association between female skimming frequency and focal male size (Figure
270 3, Table 3). Simulated data generated from our best-fit models generated distributions that
271 complied with observed data.

272

273 DISCUSSION

274 The aim of this study was to identify the cues used by *R. amarus* in responding to oviposition
275 sites. Appropriate responses to host cues are a key component of the mating system of this
276 species (Smith et al., 2004), as well as other taxa (Refsnider & Janzen, 2010). We discriminated
277 which cues were responsible for the occurrence of a response to a cue, and when a response did
278 occur, its magnitude by fitting zero-altered statistical models (Zuur et al., 2009; Hilbe, 2014).
279 Both sexes expressed a positive response to water conditioned with the odour of living mussels;
280 without this cue the reaction to artificial mussels was negligible. This response ensures that time
281 and energy are only invested in living mussels, not water flows originating from some other
282 source. Additional information may also be obtained from mussel odour cues. While *R. amarus*
283 are generalists, potentially using a range of mussel species for oviposition, other bitterling species
284 are specialists, using just one or two (Liu et al., 2006; Kitamura et al., 2012). In these cases,
285 species-specific odour cues may play a role in mussel choice (Reichard et al., 2007a) as bitterling
286 appear not to attend to visual cues that discriminate mussel species (Mills & Reynolds, 2002b).
287 Chemosensory cues are crucial in the oviposition-site decisions in other taxa, including
288 *Drosophila* spp. (Riffell, 2013), fig wasps (Hossaert-McKey et al., 1994), mosquitos (Afify &
289 Galizia, 2015) and parasitoids (Godfray, 1994). In *D. melanogaster*, research on the mechanistic
290 basis to oviposition-site decisions has demonstrated a role for specific volatile compounds that
291 activate specific neurons expressing a specific odorant receptor; thus a single dedicated olfactory
292 pathway determines oviposition choice in this species (Dweck et al., 2013). An understanding of
293 the mechanistic basis of a response to mussel odour by bitterling may provide insights into
294 interspecific variation in host specialism in these fishes and artificial mussel is an ideal tool to
295 achieve this goal.

296 Females showed a significant response to a high dissolved oxygen concentration. Oxygen
297 availability is critical to egg and embryo development and survival during incubation in the
298 mussel gill. Bitterling eggs are relatively large compared to other similarly sized fish, allowing

299 them to fit in the interlamellar spaces of a mussel gill, and consequently have a high *per capita*
300 oxygen requirement (Aldridge, 1999). Given that mussels sometimes host well over 100 bitterling
301 eggs (Smith et al., 2001; Kitamura, 2005), competition for oxygen inside the mussel gill can be
302 severe, both among embryos and between embryos and host, and it is notable that embryo
303 mortality rates in mussels are strongly density dependent (Smith et al., 2000, 2001; Agbali &
304 Smith, 2012; Spence & Smith, 2013), presumably due to asphyxiation (Aldridge, 1999;
305 Kitamura, 2006). Consequently, natural selection is predicted to favour a preference for cues that
306 indicate directly whether a mussel is hosting the eggs and embryos of other females, or indirectly
307 through the decline in quality of a mussel as a result of superparasitism. Thus the response by
308 females for mussels with high concentrations of dissolved oxygen in the exhalant flow of the
309 artificial mussel appears adaptive, indicating to a female a mussel in good condition that contains
310 few other embryos, which are potential competitors of her own offspring. Such avoidance of
311 superparasitism is particularly well understood in parasitoids (Godfray, 1994; Gandon et al.,
312 2006) where in some wasps, females make oviposition decisions associated with interspecific,
313 intraspecific and self-superparasitism via 'patch marking', chemical cues left by females during
314 oviposition (van der Hoeven & Hemerick, 1990; Viser, 1993; Harvey, 2000). Whether female
315 bitterling can detect bitterling eggs and embryos in mussel gills is not currently known, however
316 the indirect detection of superparasitism from dissolved oxygen levels in the mussel exhalant
317 flow may operate in the bitterling system.

318 While female *R. amarus* showed a significant threshold response to high dissolved oxygen
319 conditions, the strength of female response to artificial mussels was predicted by the difference
320 between the dissolved oxygen concentration of the mussel exhalant flow and the ambient oxygen
321 concentration; the lower the exhalant flow dissolved oxygen concentration relative to ambient,
322 the lower the female response (Table 3). The implication of this finding is that the female
323 response to an oxygen cue is labile and based on comparative evaluation, rather than a fixed
324 response to a threshold dissolved oxygen concentration. This outcome fits in the context of the

325 ecology of bitterling and their mating system. The spawning season of *R. amarus* is relatively
326 protracted, starting in mid-April and typically ending in mid-June (Konečná & Reichard, 2011).
327 As the spawning season advances, mussels fill with eggs and embryos and progressively decline
328 in quality as incubation sites (Kitamura, 2005; Smith, 2017). Water temperatures also
329 progressively increases, with a concomitant decline in dissolved oxygen concentration. The
330 capacity to distinguish the relative, rather than absolute, quality of an individual mussel is,
331 therefore, critical in accommodating this temporal pattern in oviposition site quality based on
332 dissolved oxygen conditions inside the mussel.

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

340 Male *R. amarus* responded to water flow velocity from artificial mussels with an elevated
341 ejaculation frequency. The approach taken in this study does not allow the framing of this
342 preference as “choice” by the males. Males here displayed a plastic response to flow by not
343 exclusively ejaculating in high flow mussels, but only in adjustments to their behaviour in
344 response to flow. This positive, plastic response by males to water flow may reflect an unusual
345 aspect of the bitterling mating system. Male *R. amarus* perform multiple ejaculations over
346 mussels, even without a female present, ejaculating over a guarded mussel on >200 occasions
347 daily under natural conditions (Smith et al., 2009). This pattern of sperm release appears to
348 function in maintaining a baseline level of spermatozoa in a mussel's gills (Smith & Reichard,
349 2013), thereby ensuring fertilization should a female oviposit in the mussel. Sperm released into a
350 mussel potentially undergoes passive loss from its gills as it filters water. The rate that males

351 ‘top-up’ mussels with sperm differs between bitterling species, and is sensitive to the presence of
352 rivals and females in spawning condition (Smith et al., 2014a). Filtration rates vary naturally
353 among (Smith et al., 2001) and within host mussel species (Mills & Reynolds, 2002b). Smith &
354 Reichard (2013) speculated that because mussels filter water at different rates (either due to
355 species or individual differences) males might be sensitive to mussel flow rate and should
356 respond to elevated flow rates by increasing ejaculation rates to keep mussels topped-up with
357 sperm (*sensu* Parker, 1998). The results of the present study support this hypothesis (Figure 3).
358 Thus while variation in mussel flow rates did not inhibit male host preference, our results
359 demonstrate that males are capable of adjusting their behaviour adaptively to their current host.

360 Male size was negatively associated with the frequency of ejaculation and also female
361 response to mussels (Table 3). Male size determines dominance in bitterling (Smith et al., 2003;
362 Casalini et al., 2009), with the largest males tending to act as guards and smaller males acting
363 as sneaks (Smith et al., 2004). This pattern is a common feature of the mating systems of fishes
364 (Wootton & Smith, 2015) and other taxa (Arnqvist & Rowe, 2005). Smaller male bitterling have
365 relatively (though not absolutely) larger testis size (Smith et al., 2014a) and typically compete
366 with rivals through sperm competition rather than direct aggressive contests (Reichard et al.,
367 2004), which may explain the higher ejaculation rate of smaller males in the present study. Male
368 bitterling increase their sperm investment through elevated frequency of ejaculation, not larger
369 ejaculate size (Candolin & Reynolds, 2002). The reason for a greater female response to smaller
370 males is unclear. Male size and dominance do not appear to play a role in female mate choice,
371 though large dominant males are typically able to monopolize mussels and thereby to achieve
372 high reproductive success (Reichard et al., 2007b, 2009; Casalini et al., 2009). Male nuptial
373 colour similarly has not been demonstrated to have a direct effect on female mate choice
374 (Reichard et al., 2005; Casalini et al., 2009). Without measuring further male traits such as
375 genetic compatibility, we are unable to account for this apparent elevated response by females to
376 smaller males.

377 *Conclusion*

378 Overall our results demonstrated that males and females responded to common, but also
379 contrasting mussel cues. Both sexes responded almost exclusively to artificial mussels with the
380 odour of living mussels, but while males failed to respond to dissolved oxygen levels, females
381 showed a response to a high dissolved oxygen concentration and large relative difference in
382 oxygen concentration between the artificial mussel and ambient. In contrast, while females did
383 not respond to differences in water flow from the artificial mussel, males responded to higher
384 flows by elevating their ejaculation rate (Table 3). These differences may reflect different
385 adaptive priorities for males and females. Thus, while females attend to cues that reflect mussel
386 quality as a site for incubation of young stages (Smith et al., 2001, 2002; Agbali et al., 2010;
387 Agbali & Smith, 2012), males instead appear sensitive to the risk of sperm competition (Spence,
388 Reichard & Smith, 2013), and are insensitive to mussel quality (Smith et al., 2002, 2003, 2014b;
389 Casalini et al., 2013). A sexual conflict over responses to oviposition sites in *R. amarus* may,
390 consequently, arise. Sexual conflict occurs when the evolutionary interests of individuals of the
391 two sexes diverge (Parker, 1979), with a potential to generate sexually antagonistic selection
392 (Lessells, 2006). In the context of the bitterling mating system, responses to oviposition-site cues
393 are a potential arena for sexual conflict, with females maximizing offspring fitness through
394 attending to the dissolved oxygen concentration of water emerging from the mussel exhalant
395 siphon, and males maximizing fertilization success through sperm competition by responding to
396 water flow velocity and the behaviour of rivals. These differences appear to manifest themselves
397 as overt behavioural conflicts between spawning partners (Smith et al., 2002). Over the course of
398 a spawning event males repeatedly attempt to lead females away from mussels with nearby rivals
399 while females frequently select alternative mussels on the basis of offspring survival. We are
400 aware of no other mating system with conflicting responses to oviposition-site cues like that seen
401 in *R. amarus*.

402 REFERENCES

- 403 Adolph, E.F. (1920). Egg-laying reactions in the pomace fly, *Drosophila*. Journal of
404 *Experimental Zoology*, 31, 326-341.
- 405 Afify, A. & Galizia, C.G. (2015). Chemosensory cues for mosquito oviposition site selection.
406 *Journal Medical Entomology*, 52, 120-130.
- 407 Agbali, M., Reichard, M., Bryjová, A., Bryja, J. & Smith, C. (2010). Mate choice for nonadditive
408 genetic benefits correlate with MHC dissimilarity in the rose bitterling (*Rhodeus ocellatus*).
409 *Evolution*, 64, 1683-1696.
- 410 Agbali, M. & Smith, C. (2012). Long-term fitness consequences for offspring of female
411 oviposition decisions in the rose bitterling (*Rhodeus ocellatus*). *Acta Zoologica*, 93, 367-372.
- 412 Akaike, H. (1973). Maximum likelihood identification of Gaussian autoregressive moving
413 average models. *Biometrika*, 60, 255-265.
- 414 Aldridge, D.C. (1999). Development of European bitterling in the gills of freshwater mussels.
415 *Journal of Fish Biology*, 54, 138-151.
- 416 Arnqvist, G. & Rowe, L. (2005). *Sexual conflict*. Princeton, NJ: Princeton University Press.
- 417 Bernier-Bourgault, I. & Magnan, P. (2002). Factors affecting redd site selection, hatching, and
418 emergence of brook charr, *Salvelinus fontinalis*, in an artificially enhanced site. *Environmental*
419 *Biology of Fishes*, 64, 333-341.
- 420 Brabrand, Å., Koestler, A.G. & Borgstrøm, R. (2002). Lake spawning of brown trout related to
421 groundwater influx. *Journal of Fish Biology*, 60, 751-763.
- 422 Candolin, U., & Reynolds, J. D. (2001). Sexual signaling in the European bitterling: females
423 learn the truth by direct inspection of the resource. *Behavioral Ecology*, 12(4), 407-411.
- 424 Candolin, U., & Reynolds, J. D. (2002). Adjustments of ejaculation rates in response to risk of
425 sperm competition in a fish, the bitterling (*Rhodeus sericeus*). *Proceedings of the Royal*
426 *Society of London B: Biological Sciences*, 269(1500), 1549-1553.
- 427 Casalini, M., Agbali, M., Reichard, M., Konečná, M., Bryjová, A. & Smith, C. (2009). Male
428 dominance, female mate choice, and intersexual conflict in the rose bitterling (*Rhodeus*
429 *ocellatus*). *Evolution*, 63, 366-376.
- 430 Casalini, M., Reichard, M., Phillips, A. & Smith, C. (2013). Male choice of mates and mating
431 resources in the rose bitterling (*Rhodeus ocellatus*). *Behavioural Ecology*, 24, 1199-1204.

- 432 Chapman, D.W. (1988). Critical review of variables used to define effects of fines in redds of
433 large salmonids. *Transactions of the American Fisheries Society*, 117, 1–21.
- 434 Clutton-Brock, T.H. (1991). *The evolution of parental care*. Princeton, NJ: Princeton University
435 Press.
- 436 Cope, J.M. & Fox, C.W. (2003). Oviposition decisions in the seed beetle, *Callosobruchus*
437 *maculatus* (Coleoptera: Bruchidae): effects of seed size on superparasitism. *Journal of Stored*
438 *Products Research*, 39, 355-365.
- 439 Córdoba-Aguilar, A. (2009). A female evolutionary response when survival is at risk: male
440 harassment mediates early reallocation of resources to increase egg number and size.
441 *Behavioural Ecology and Sociobiology*, 63, 751-763.
- 442 Davenport, J. & Woolmington, A.D. (1982). A new method of monitoring ventilatory activity in
443 mussels and its use in a study of the ventilatory patterns of *Mytilus edulis* L. *Journal of*
444 *Experimental Marine Biology and Ecology*, 62, 55-67.
- 445 Dweck, H.K., Ebrahim, S.A., Kromann, S., Bown, D., Hillbur, Y., Sachse, S., et al. (2013).
446 Olfactory preference for egg laying on citrus substrates in *Drosophila*. *Current Biology*, 23,
447 2472-2480.
- 448 Esteve, M. (2005). Observations of spawning behaviour in Salmoninae: *Salmo*, *Oncorhynchus*
449 and *Salvelinus*. *Reviews of Fish Biology and Fisheries*, 15, 1–21.
- 450 Gandon, S., Rivero, A. & Varaldi, J. (2006). Superparasitism evolution: adaptation or
451 manipulation? *American Naturalist*, 167, E1-E22.
- 452 Godfray, H.C.J. (1994). *Parasitoids*. Princeton, NJ: Princeton University Press
- 453 Harvey, J.A. (2000). Dynamic effects of parasitism by an endoparasitoid wasp on the
454 development of two host species: implications for host quality and parasitoid fitness.
455 *Ecological Entomology*, 25, 267-278.
- 456 Heschl, A. (1989). Integration of “innate” and “learned” components within the IRME for mussel
457 recognition in the European bitterling *Rhodeus amarus* (Bloch). *Ethology*, 81, 193-208.
- 458 Hilbe, J.M. (2014). *Modeling count data*. Cambridge, UK: Cambridge University Press.
- 459 Hossaert-McKey, M., Gibernau, M. & Frey, J.E. (1994). Chemosensory attraction of fig wasps to
460 substances produced by receptive figs. *Entomologia Experimentalis et Applicata*, 70: 185-
461 191.

462 Ieno, E.N. & Zuur, A.F. (2015). Data exploration and visualisation with R. Highland Statistics
463 Ltd, Newburgh.

464 Zuur, A. F., & Ieno, E. N. (2016). A protocol for conducting and presenting results of regression-
465 type analyses. *Methods in Ecology and Evolution*, 7(6), 636-645.

466 Jackman, S. (2014). pscl: classes and methods for R developed in the political science
467 computational laboratory, Stanford University (R package version 1.4.6). Stanford University,
468 Stanford, CA.

469 Janáč, M. (2009) *Sampling and evaluation of the young-of-the-year fish assemblages* (Doctoral
470 dissertation). Brno, Czech Republic: Masarykova Univerzita

471 Kitamura, J. (2005). Factors affecting seasonal mortality of rosy bitterling (*Rhodeus ocellatus*
472 *kurumeus*) embryos on the gills of their host mussel. *Population Ecology*, 47, 41-51.

473 Kitamura, J. (2006). Adaptive spatial utilization of host mussels by the Japanese rosy bitterling
474 *Rhodeus ocellatus kurumeus*. *Journal of Fish Biology*, 69, 263-271.

475 Kitamura, J., Nagata, N., Nakajima, J. & Sota, T. (2012). Divergence of ovipositor length and egg
476 shape in a brood parasitic bitterling fish through the use of different mussel hosts. *Journal of*
477 *Evolutionary Biology*, 25, 566-573.

478 Konečná, M. & Reichard, M. (2011). Seasonal dynamics in population characteristics of
479 European bitterling *Rhodeus amarus* in a small lowland river. *Journal of Fish Biology*, 78,
480 227-239.

481 Lessells, C.M. (2006). The evolutionary outcome of sexual conflict. *Philosophical transactions*
482 *of the Royal Society of London. Series B, Biological sciences*, 361, 301-317.

483 Liu, H., Yurong, Z., Reichard, M. & Smith, C. (2006). Evidence of host specificity and
484 congruence between phylogenies of bitterlings and freshwater mussels. *Zoological Studies*, 45,
485 428-434.

486 Mayhew, P.J. (1997). Adaptive patterns of host-plant selection by phytophagous insects. *Oikos*,
487 79, 417-428.

488 Mazerolle, M.J. (2016). AICcmodavg: model selection and multimodel inference based on
489 (Q)AIC(c). R package version 2.1-0.

490 Mills, S.C. & Reynolds, D.C. (2002a). Host species preferences by bitterling, *Rhodeus sericeus*,
491 spawning in freshwater mussels and consequences for offspring survival. *Animal Behaviour*,
492 63, 1029-1036.

- 493 Mills, S.C. & Reynolds, D.C. (2002b). Mussel ventilation rates as a proximate cue for host
494 selection by bitterling, *Rhodeus sericeus*. *Oecologia*, 131, 473-478.
- 495 Mousseau, T.A. & Fox, C.W. (1998). The adaptive significance of maternal effects. *Trends in*
496 *Ecology and Evolution*, 13, 403-407.
- 497 Parker, G.A. (1979). Sexual selection and sexual conflict. — In: Sexual selection and
498 reproductive competition in insects (Blum, M.S. & Blum, N.A., eds). Academic Press, New
499 York, p. 123-166.
- 500 Parker, G.A. (1998). Sperm competition and the evolution of ejaculates: towards a theory base. In
501 Birkhead, T.R. & Møller, A.P., (Eds.), *Sperm competition and sexual selection*. (pp. 3 -54),
502 London, UK: Academic Press.
- 503 Payne, A.G., Smith, C. & Campbell, A.C. (2002). Filial cannibalism improves survival and
504 development of beaugregory damselfish embryos. *Proceedings of the Royal Society of London*
505 *B: Biological Sciences*, 269, 2095-2102.
- 506 Qvarnström, A. & Forsgren, E. (1998). Should females prefer dominant males? *Trends in*
507 *Ecology and Evolution*, 13, 498-501.
- 508 R Development Core Team (2016). R: a language and environment for statistical computing. R
509 Foundation for Statistical Computing, Vienna, available online at <http://www.R-project.org/>.
- 510 Refsnider, J.M. & Janzen, F.J. (2010). Putting eggs in one basket: ecological and evolutionary
511 hypotheses for variation in oviposition-site choice. *Annual Reviews of Ecology, Evolution and*
512 *Systematics*, 41, 39-57.
- 513 Reichard, M., Smith, C. & Jordan, W.C. (2004). Genetic evidence reveals density-dependent
514 mediated success of alternative mating behaviours in the European bitterling (*Rhodeus*
515 *sericeus*). *Molecular Ecology*, 13, 1569-1578.
- 516 Reichard, M., Bryja, J., Ondračková, M., Dávidová, M., Kaniewska, P., & Smith, C. (2005).
517 Sexual selection for male dominance reduces opportunities for female mate choice in the
518 European bitterling (*Rhodeus sericeus*). *Molecular Ecology*, 14(5), 1533-1542.
- 519 Reichard, M., Ondračková, M., Przybylski, M., Liu, H. & Smith, C. (2006). The costs and
520 benefits in an unusual symbiosis: experimental evidence that bitterling fish (*Rhodeus sericeus*)
521 are parasites of unionid mussels in Europe. *Journal of Evolutionary Biology*, 19, 788-796.

- 522 Reichard, M., Liu, H. & Smith, C. (2007a). The co-evolutionary relationship between bitterling
523 fishes and freshwater mussels: insights from interspecific comparisons. *Evolutionary Ecology*
524 *Research*, 9, 239-259.
- 525 Reichard, M., LeComber, S.C. & Smith, C. (2007b). Sneaking from a female perspective. *Animal*
526 *Behaviour*, 74, 679-688.
- 527 Reichard, M., Ondračková, M., Bryjová, A., Smith, C., & Bryja, J. (2009). Breeding resource
528 distribution affects selection gradients on male phenotypic traits: experimental study on
529 lifetime reproductive success in the bitterling fish (*Rhodeus amarus*). *Evolution*, 63(2), 377-
530 390.
- 531 Riffell, J.A. (2013). Neuroethology: lemon-fresh scent makes flies lay eggs. *Current Biology*, 23,
532 R1108-R1110.
- 533 Roitberg, B.D. (1998). Oviposition decisions as maternal effects: conundrums and opportunities
534 for conservation biologists. In Mousseau, T.A. & Fox, C.W., (Eds.) *Maternal effects as*
535 *adaptations* (p. 67-81). Oxford, UK: Oxford University Press.
- 536 Shine, R. & Harlow, P.S. (1996). Maternal manipulation of offspring phenotypes via nest-site
537 selection in an oviparous lizard. *Ecology*, 77, 1808-1817.
- 538 Smith, C., Reynolds, J.D., Sutherland, W.J. & Jurajda, P. (2000). Adaptive host choice and
539 avoidance of superparasitism in the spawning decisions of bitterling (*Rhodeus sericeus*)
540 *Behavioural Ecology and Sociobiology*, 48, 29-35.
- 541 Smith, C., Rippon, K., Douglas, A. & Jurajda, P. (2001). A proximate cue for oviposition site
542 choice in the bitterling (*Rhodeus sericeus*). *Freshwater Biology*, 46, 903-911.
- 543 Smith, C., Douglas, A. & Jurajda P. (2002). Sexual conflict, sexual selection and sperm
544 competition in the spawning decisions of bitterling, *Rhodeus sericeus*. *Behavioural Ecology*
545 *and Sociobiology*, 51, 433-439.
- 546 Smith, C., Reichard, M. & Jurajda, P. (2003). Assessment of sperm competition by European
547 bitterling, *Rhodeus sericeus*. *Behavioural Ecology and Sociobiology*, 53, 206-213.
- 548 Smith, C., Reichard, M., Jurajda, P. & Przybylski, M. (2004). The reproductive ecology of the
549 European bitterling (*Rhodeus sericeus*). *Journal of Zoology*, 262, 107-124.
- 550 Smith, C. & Reichard, M. (2005). Females solicit sneakers to improve fertilization success in the
551 bitterling fish (*Rhodeus sericeus*). *Proceedings of the Royal Society of London B: Biological*
552 *Sciences*, 272, 1683-1688.

- 553 Smith, C., Pateman-Jones, C., Zięba, G., Przybylski, M. & Reichard, M. (2009). Sperm depletion
554 as a consequence of increased sperm competition risk in the European bitterling, *Rhodeus*
555 *amarus*. *Animal Behaviour*, 77, 1227-1233.
- 556 Smith, G. & Ritchie, M.G. (2013). How might epigenetics contribute to ecological speciation?
557 *Current Zoology*, 59, 686-696.
- 558 Smith, C. & Reichard, M. (2013). A sperm competition model for the European bitterling
559 (*Rhodeus amarus*). *Behaviour*, 150, 1709-1730.
- 560 Smith, C., Warren, M., Rouchet, R. & Reichard, M. (2014a). The function of multiple
561 ejaculations in bitterling. *Journal of Evolutionary Biology*, 27, 1819-1829.
- 562 Smith, C., Phillips, A., Polačik, M. & Reichard, M. (2014b). Male coloration signals direct
563 benefits in the European bitterling (*Rhodeus amarus*). *Environmental Biology of Fishes*, 97,
564 335-341.
- 565 Smith, C., Phillips, A. & Reichard, M. (2015). Cognitive ability is heritable and predicts the
566 success of an alternative mating tactic. *Proceedings of the Royal Society of London B:*
567 *Biological Sciences*, 282, 20151046.
- 568 Smith, C. (2017). Bayesian inference supports the host selection hypothesis in explaining
569 adaptive host specificity by European bitterling. *Oecologia*, in press
- 570 Soler, M. (2014). Long-term coevolution between avian brood parasites and their hosts.
571 *Biological Reviews*, 89, 688-704.
- 572 Spence, R. & Smith, C. (2005). Male territoriality mediates density and sex ratio effects on
573 oviposition in the zebrafish, *Danio rerio*. *Animal Behaviour*, 69, 1317-1323.
- 574 Spence, R., Reichard, M. & Smith, C. (2013). Strategic sperm allocation and a Coolidge effect in
575 an externally fertilizing species. *Behavioural Ecology*, 24, 82-88.
- 576 Spence, R. & Smith, C. (2013). Rose bitterling (*Rhodeus ocellatus*) embryos parasitize freshwater
577 mussels by competing for nutrients and oxygen. *Acta Zoologica*, 94, 113-118.
- 578 Stadnichenko, A.P. & Stadnichenko, Y.A. (1980). On the effect of bitterling larvae on the
579 lamellibranchid mollusc *Unio rostratus gentilis* Haas. *Gidrobiologicheski Zhurnal*, 1980, 57-
580 61.
- 581 Taylor, A.J., Müller, C.B. & Godfray, H.C.J. (1998). Effect of aphid predators on oviposition
582 behavior of aphid parasitoids. *Journal of Insect Behaviour*, 11, 297-302.
- 583 Tinbergen, N. (1951). *The study of instinct*. Oxford, UK: Oxford University Press

- 584 van der Hoeven, N. & Hemerik, L. (1990). Superparasitism as an ESS: to reject or not to reject,
585 that is the question. *Journal of Theoretical Biology*, 146, 467-482.
- 586 Viser, M.E. (1993). Adaptive self-and conspecific superparasitism in the solitary parasitoid
587 *Leptopilina heterotoma* (Hymenoptera: Eucoilidae). *Behavioural Ecology*, 4, 22-28.
- 588 Wiepkema, P.R. (1961). An ethological analysis of the reproductive behaviour of the bitterling
589 (*Rhodeus amarus* Bloch). *Archives Néerlandaises de Zoologie*, 14, 103-199.
- 590 Wolfner, M.F. (2002). The gifts that keep on giving: physiological functions and evolutionary
591 dynamics of male seminal proteins in *Drosophila*. *Heredity*, 88, 85-93.
- 592 Wootton, R.J. & Smith, C. (2015). *Reproductive biology of teleost fishes*. Oxford, UK: Wiley-
593 Blackwell
- 594 Zuur, A., Ieno, E.N., Walker, N., Saveliev, A.A. & Smith, G.M. (2009). *Mixed effects models and*
595 *extensions in ecology with R*. New York: Springer

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

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

597

598 **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/l
	Low	1.5 mg/l
Mussel odour	Present	80 mussels/m ³
	Absent	0 mussels/m ³

599

601 **Table 3.** Results of best-fit zero-altered negative binomial (ZANB)^a and zero-altered Poisson
602 (ZAP)^b models testing the effects of mussel parameters on the responses of male and female *R.*
603 *amarus*. Bold text indicates significant results to P < 0.05.

Sex	Parameter	Occurrence model				Frequency model			
		γ	SE	Z	P	β	SE	Z	P
Male ^a	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 ^b	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

604

605 **Figure 1**

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

608 **Figure 2**

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

613 **Figure 3**

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





