1 **Avoidance of host resistance in the oviposition-site preferences of rose bitterling**

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Abstract A contemporary outcome of dynamic host-parasite coevolution can be driven by the adaptation of a parasite to exploit its hosts at the population and species levels (parasite specialisation) or by local host adaptations leading to greater host resistance to sympatric parasite populations (host resistance). We tested the predominance of these two scenarios using cross-infection experiments with two geographically distant populations of the rose bitterling, *Rhodeus ocellatus*, a fish brood parasite of freshwater mussels, and four populations of their mussel hosts (two *Anodonta woodiana* and two *Unio douglasiae* populations) with varying degrees of geographic sympatry and local coexistence. Our data support predictions for host resistance at the species level but no effect of local coexistence between specific populations. *Rhodeus ocellatus* showed a preference for allopatric host populations, irrespective of host species. Host mussel response, in terms of ejection of *R. ocellatus* eggs, was stronger in the more widespread and abundant host species (*A. woodiana*) and this response tended to be higher in sympatric populations. These outcomes provide support for the importance of host resistance in bitterling oviposition-site decisions, demonstrating that host choice by *R. ocellatus* is adaptive by minimizing egg ejections. These findings imply that *R. ocellatus*, and potentially other bitterling species, may benefit from exploiting novel hosts, which may not possess appropriate adaptive responses to parasitism.

Introduction

Host-parasite relationships impose strong selective forces in evolution (Woolhouse et al. 2002). The intensity of selection resulting from biological interactions can vary over space and time, ranging from hotspots, with species exhibiting rapid reciprocal adaptations, to coldspots where the same species do not coevolve despite their co-occurrence (Laine 2009). Differences in selective environments may favour different traits in different interacting populations, leading to distinct levels of reciprocal selection, as predicted by the geographic mosaic theory of coevolution (Thompson 1999, 2013).

Most host-parasite relationships incur significant costs to both partners and result in a rapid coevolutionary arms race when an adaptation in one partner is followed by reciprocal counter-adaptations in the other (Dawkins and Krebs 1979). This process is dynamic and at a particular evolutionary point, hosts may be better adapted to defend themselves from the parasite or, alternatively, the parasite can be better adapted to exploit its host (Rothstein and Robinson 1998; Sorensen et al. 2004; Medina and Langmore 2016). *The parasite specialization scenario* considers that the present coevolutionary state is primarily resolved by traits evolved by the parasite, with parasite adaptations currently overcoming the defences evolved by the host. The *host resistance scenario* predicts that the present coevolutionary state is primarily resolved by the host, which expresses adaptations that resist parasite virulence (Davies and Brooke 1988; Honza et al. 2004). An alternative possibility is that adaptive responses in the host-parasite relationship are lacking, a situation predicted when the cost, probability or intensity of an interaction is limited (Holland et al. 2004).

Parasites may specialise on particular host species (Joshi and Thompson 1995) or specific host populations (Kaltz and Shykoff 1998; Kawecki and Ebert 2004). Host communities vary in

63 their species composition, relative abundance of each host species and density of their

64 populations. Given that each host species may require different parasite adaptations, variation in

65 the structure of local host communities may lead to *parasite specialization at the local level*,

66 often to the locally most common host species (Thompson 1994, 1999; Krasnov et al. 2011).

67 This situation produces variable host-parasite associations across geographic regions, leading to

68 local differences in parasite specialization. An example is the European cuckoo (*Cuculus*

69 *canorus* L.) where different populations specialize on different locally accessible host species

70 (Medina and Langmore 2016).

71   In other host-parasite systems, however, parasites may coevolve with a single host

72 species across their entire range. *Parasite specialization at the species level* requires generalised

73 adaptations to exploit a host species and may be favoured when either parasite or host species

74 disperse over large distances (Medina and Langmore 2016). In African indigobirds (*Vidua* spp.),

75 strict host-parasite association at the species level is driven by precise mouth marking in chicks

76 that mimic mouth marking of their respective host species (Sorenson et al. 2003). Stronger

77 specialization on one host species, therefore, comes at a cost of a decrease in fitness on other

78 host species, leading to parasite specialisation at the level of the host species. High host

79 specificity is expected to lead to more intense escalation of reciprocal adaptations in hosts and

80 parasites.

81   An analogous, though reversed, scenario is the situation when host resistance dominates

82 the current state of the reciprocal relationship. Increased resistance to parasites can improve a

83 host's fitness, but is often costly as resources are diverted from growth and reproduction (Moret

84 and Schmid-Hempel 2000; Rigby and Moret 2000; Schmid-Hempel 2003). Hence, a high

85 investment in host resistance is more likely to be maintained under intense parasite pressure.

86 When effective host responses are detected, *host resistance* can be defined at the species and

87 local population levels, similarly to parasite specialization.

88   A powerful way to test for host and parasite adaptation is by using cross-infection

89 experiments to compare parasite success (or preference) and host resistance for sympatric versus

90 allopatric populations (reviewed by Kawecki and Ebert 2004). *Local parasite adaptation* can be

91 detected by measuring the fitness of a parasite on locally coexisting host populations, with a

92 prediction of parasite preference and increased fitness on locally coexisting hosts. In contrast,

93 *local host resistance* predicts increased parasite fitness on ecologically naive hosts and, in the

94 case that the parasite is capable of evaluating host suitability, parasite preference for ecologically

95 naive host populations over coexisting host populations (Kawecki and Ebert 2004). Previous

96 studies have provided evidence for local adaptation of the parasite, local adaptation of the host,

97 and no local adaptation (Edmunds and Alstad 1978; Greischar and Koskella 2007; Hoeksema

98 and Forde 2008; Laine 2009; Voutilainen et al. 2009).

99   In this study, we investigated interpopulation variation in host-parasite association

100 between a parasitic fish, the rose bitterling, *Rhodeus ocellatus* (Kner), and their host mussels

101 from the family Unionidae. *Rhodeus ocellatus*, like all other bitterling fishes (family

102 Acheilognathidae), lay their eggs in the gills of unionid mussels. Female bitterling use a long

103 ovipositor to lay their eggs in the gills of living mussels by inserting them into the host's exhalant

104 siphon. Male bitterling fertilize the eggs deposited inside the mussel gill by releasing sperm over

105 the host inhalant siphon (Reichard et al. 2004a; Smith and Reichard 2013). Males defend a

106 territory containing one or more mussels against rivals, actively court females and lead them to a

107 mussel (Smith et al. 2004). Bitterling embryos develop in the gills of the host mussel, competing

108 for nutrients and oxygen with the host, before emerging after 3-4 weeks (Aldridge 1999; Spence

109   and Smith 2013). Hosting bitterling embryos is costly to mussels (Reichard et al. 2006), and

110   mussels have evolved counter-adaptations to bitterling parasitism, primarily ejections of their

111   eggs and embryos (Kitamura 2005; Reichard et al. 2007; Reichard et al. 2010). In turn, bitterling

112   eggs and embryos have evolved behavioural and morphological mechanisms to avoid ejection

113   (Smith et al. 2004). The bitterling-mussel system is conceptually analogous to avian brood

114   parasitism (Davies 2016).

115   *Rhodeus ocellatus* is widely distributed across East Asia (Chang et al. 2014). It is a

116   generalist parasite utilising a range of host mussel species, but exhibiting preferences for

117   particular hosts (Reichard et al. 2007). Female *R. ocellatus* display preferences toward certain

118   mussel individuals (Casalini et al. 2013), indicating that female *R. ocellatus* may tailor their host

119   preferences to host quality cues. At least at the level of individual host mussels, males appear

120   less selective than females (Casalini et al. 2013), trading off mussel quality against the risk of

121   sperm competition (Smith et al. 2003). Although female oviposition decisions are also affected

122   by mate quality (Agbali et al. 2010, 2012), the quality of the host mussel is the key factor

123   predicting female oviposition-site decisions (Casalini et al. 2009). Female host choice decisions

124   in *R. ocellatus* and other bitterling species rely on optimal mussel traits related to enhanced

125   embryo survival, particularly the availability of oxygen for developing embryos (Smith et al.

126   2001; Reichard et al. 2007; Spence and Smith 2013; Phillips et al. 2017).

127   There is good evidence for coevolutionary dynamics in the relationship between

128   bitterling fishes and their mussel hosts. Populations of the related European bitterling, *Rhodeus*

129   *amarus* (Bloch)*,* display variation in host species preference across their geographic distribution

130   (Reichard et al. 2010; Smith 2017). Similarly, host mussels have evolved stronger defences (e.g.

131   egg ejection) in areas of ancient sympatry with *R. amarus* than in areas of recent sympatry

132   (Reichard et al. 2010). Hence, bitterling and their hosts can exhibit population-level variation in

133   the intensity of reciprocal selection. We hypothesized that this variation may lead to different

134   degrees of local adaptation, especially in areas of ancient sympatry, driven either by parasite

135   adaptation or host resistance.

136   Here, we used two geographically distant populations of *R. ocellatus* from a region of

137   ancient bitterling-mussel sympatry to test their preference towards two widespread host mussel

138   species, *Anodonta woodiana* (Lea) and *Unio douglasiae* (Gray). Despite wide co-occurrence

139   between both mussel host species and *R. ocellatus*, *A. woodiana* hosts have a larger distribution

140   and are locally more abundant, typically being the dominant unionid species in mussel

141   communities throughout the range of *R. ocellatus* (He and Zimin 2013). Each host mussel

142   species was represented by one population coexisting with and one population naïve to *R.*

143   *ocellatus*. Mussel host populations varied in their level of regional and local coexistence with *R.*

144   *ocellatus*, producing a mosaic of associations at the species and local levels (Fig. 1). We also

145   measured population-specific host mussel resistance to *R. ocellatus* oviposition through egg

146   ejections.

147   The coevolutionary relationship between bitterling and their mussel hosts is antagonistic

148   and dynamic (Smith et al. 2004; Reichard et al. 2012; Spence and Smith 2013). For *parasite*

149   *specialisation* to play a dominant role in the association the prediction is that *R. ocellatus* will

150   make oviposition-site decisions that favour the utilization of mussels with which there is an

151   evolutionary association at a meta (species) level, as well as at a local (population) level. In

152   contrast, in the case that *host resistance* plays the primary role in oviposition-site decisions, *R.*

153   *ocellatus* are predicted to use allopatric mussel species at a meta level and populations without

154   previous exposure to bitterling at a local level. In the context of the present study, in the case of

155   *parasite specialisation at the species level* we predicted a preference by experimental *R.*

156   *ocellatus* for sympatric host species at a broad (meta) geographic scale and, under the *parasite*

157   *specialisation at the local level*, a preference for populations with which they locally coexist. In

158   the case of *host resistance at the species level* we predicted a preference for allopatric and locally

159   naive hosts. These predictions assumed that *R. ocellatus* are able to assess relevant cues

160   associated with a host mussel's ability to eject eggs as the European bitterling *R. amarus* do

161   (Mills and Reynolds 2002), though we did not test this assumption. Predictions for each host

162   mussel and bitterling population under each coevolutionary scenario are summarized in Table 1.

163

164   **Material and methods**

165   **Study subjects**

166   Two species of host mussel (*Anodonta woodiana* and *Unio douglasiae*) were collected from each

167   mussel collection site. These were Lake Bao'an in Hubei Province (30° 17' 25.4" N; 114° 43'

168   48.9" E) and Lake Qinglan (28° 30' 40.144" N; 116° 8' 2.112" E), Jiangxi Province, China. Both

169   mussel species were confirmed to be hosts of *R. ocellatus* (Reichard et al. 2007; R. Rouchet

170   unpublished data). Mussels locally coexisting with the local *R. ocellatus* population were

171   collected from Lake Bao'an, a shallow lake with abundant aquatic vegetation where *R. ocellatus*

172   is common and co-occurs with at least four other bitterling species - *Rhodeus sinensis* Guenther,

173   *Rhodeus fangi* (Miao), *Acheilognathus chankaensis* (Dybowski) and *Acheilognathus imberbis*

174   Guenther. Mussels naive to *R. ocellatus* were collected from Lake Qinglan, a part of Lake

175   Poyang, the largest lake in China. Bitterling species recorded in Lake Qinglan include *A.*

176   *chankaensis*, *A. imberbis, Acheilognathus tonkinensis* (Vaillant), *Acheilognathus* cf. *meridianus*

177   (Wu), *Acheilognathus barbatulus* Guenther, *Acheilognathus rhombeus* (Temminck & Schlegel)

178   and *Acheilognathus gracilis* Nichols. There are no records of *R. ocellatus* from the lake although

179   this species is found in some smaller affiliated waters. Given the pronounced dispersal capability

180   of unionid mussels during their larval stage (Douda et al. 2012), it is possible that host mussels

181   from Lake Qinglan may have a history of coexistence with *R. ocellatus* at a larger scale, despite

182   individual host mussels from Lake Qinglan not co-occurring with *R. ocellatus*. In Lake Qinglan,

183   *U. douglasiae* is known to be parasitized by the embryos of *A. tonkinensis* (a bitterling species

184   unrelated to *R. ocellatus*), while there are no records of bitterling embryos recovered from *A.*

185   *woodiana* from Lake Qinglan (Liu et al. 2006).

186   Mussels were collected by hand from both lakes. In the case of Lake Qinglan, because of

187   its greater depth, specimens were also collected using a mussel dredge hauled by a boat. Stocks

188   of experimental *A. woodiana* and *U. douglasiae* were collected in early April 2014 and

189   supplemented during three additional collections in Lake Bao'an and four in Lake Qinglan

190   between late April and mid-June. Mussels were stored in large (2.0 x 1.0 m) plastic containers

191   filled with water to a depth of approximately 300 mm and aerated continuously.

192   *Rhodeus ocellatus* used in the study were collected in April and May 2014 using baited

193   fish traps. Fish from the locally coexisting population were collected from Lake Bao'an (the

194   same site as mussel collection) where *R. ocellatus* lived in sympatry with at least four species of

195   freshwater mussel, including both study host species (included as two host study populations).

196   The second population of *R. ocellatus* (Nanchang) was not coexisting with any experimental

197   populations of host mussels, but coexisted with a local (non-experimental) population of *A.*

198   *woodiana*. Fish from this *R. ocellatus* population were collected in a natural pond in the city of

199   Nanchang (Jiangxi province, 28° 39' 55.08" N; 115° 48' 57.79" E), located 35 km from Lake

200   Qinglan where the mussels ecologically naive to *R. ocellatus* were collected. The pond was

201 shallow (<1 m) and measured approximately 15 x 40 m. There was no connection allowing fish

202 migration between the pond and Lake Qinglan or adjacent bodies of water. Only a single mussel

203 species (*A. woodiana*) was recorded in Nanchang pond despite an intensive search. *R. ocellatus*

204 coexisted in the pond with another bitterling species, *R. sinensis*.

205   Experimental bitterling were transported in aerated containers and housed in four large

206 (2.0 x 1.0 m) tanks. A maximum of 150 fish were stored in each tank, with approximately equal

207 sex ratio. In each tank we placed *Lamprotula caveata* (Heude) mussels to encourage female

208 ovulation. These mussels were enclosed with transparent covers that permitted bitterling to see

209 and smell them but prevented oviposition. *L. caveata* was chosen because it is phylogenetically

210 and morphologically unrelated to *A. woodiana* and *U. douglasiae* (Liu et al. 2006) and hence

211 prevented fish habituation towards one of the tested host mussels prior to the start of the

212 experiment.

213 **Behavioural experiment**

214 The experiment consisted of a test of male and female *R. ocellatus* towards a pair of host mussel

215 species of different combinations of host species, meta and local co-occurrence (Fig. 1). We use

216 the terms *sympatric* and *allopatric* at the "meta" scale (Bao'an host mussels sympatric with *R.*

217 *ocellatus*, Qinglan host mussels allopatric with *R. ocellatus*) and the terms *locally coexisting* and

218 *ecologically naïve* at the "local" scale (*ecologically naïve*: populations of fish and mussels not

219 locally coexisting regardless of sympatry at the meta scale) (Fig. 1). The experiment took place

220 between April and June 2014 in the laboratory facilities of the Institute of Hydrobiology of the

221 Chinese Academy of Sciences in Wuhan, China.

222   For each replicate, we used a set of four mussels: one *A. woodiana* from Lake Bao'an,

223 one *A. woodiana* from Lake Qinglan, one *U. douglasiae* from Lake Bao'an and one *U.*

224   *douglasiae* from Lake Qinglan (Fig. 1). Although it was impossible to obtain all four adult

225   mussels of the same size because *A. woodiana* (shell length: 91-132 mm) is larger than *U.*

226   *douglasiae* (37-91 mm), mussels from the same species within a set were size-matched. Four

227   separate preference tests (always containing a pair of host mussels; Fig. 1) were performed in a

228   random predetermined order. A full set of preference tests within a replicate was tested within

229   one day for one of the two *R. ocellatus* populations (Bao'an or Nanchang). The same set of

230   mussels was tested with the second fish population on a subsequent day, usually the following

231   day, but always within one week. A set of four host mussels (one individual from each study

232   population) was, therefore, tested in four separate paired tests (*A. woodiana* Bao'an x *A.*

233   *woodiana* Qinglan; *U. douglasiae* Bao'an x *U. douglasiae* Qinglan; *A. woodiana* Bao'an x *U.*

234   *douglasiae* Bao'an; *A. woodiana* Qinglan x *U. douglasiae* Qinglan) with the same pair of *R.*

235   *ocellatus* fish in one day and with another pair of *R. ocellatus* (from a different population) on a

236   later day (Fig. 1). In seven cases, mussel mortality prevented completion of the test with the

237   second bitterling population.

238   Experimental aquaria measured 550 (length) x 350 (width) x 450 (depth) mm and

239   contained a 20 mm layer of washed sand as a substrate. In the evening prior to the start of a test,

240   a focal male *R. ocellatus* was introduced into an experimental aquarium with a non-experimental

241   *L. caveata* mussel placed in the centre of the aquarium to elicit territoriality. On the following

242   morning the *L. caveata* mussel was removed. A second, non-experimental *R. ocellatus* male

243   (from the same population as the focal male) was placed in the tank in a cylindrical glass jar

244   (diameter 100 mm) to further stimulate the territorial and reproductive behaviour of the focal

245   male. A female in spawning condition, obvious from an extended ovipositor that unambiguously

246   demonstrates that a female bitterling has ovulated a batch of eggs, from the same population as

247   the focal male was gently released into the aquarium. The first pair of mussels to be tested were

248   placed 150 mm apart in the centre of the aquarium inside sand-filled plastic pots to hold them in

249   position. The mussels were covered with a transparent pierced plastic box that allowed the fish to

250   see and smell the mussels but prevented oviposition. After at least 30 min. of acclimation, the

251   covers were removed from the mussels. Behaviour recording started once one of the

252   experimental fish began inspection of the siphons of a mussel. Behaviours directed at a specific

253   host mussel and relevant to oviposition preference were recorded. These were: male *leading* the

254   female *R. ocellatus* towards one of the mussels (though with no interference with that mussel),

255   male or female *inspection* of the exhalant and inhalant siphons of the host mussel (sampling host

256   cues to assess its suitability for the incubation of *R. ocellatus* embryos), *sperm release* by the *R.*

257   *ocellatus* male (indicating investment into a particular host mussel, clearly detected by typical

258   male movement over the mussel inhalant siphon), *skimming* by a *R. ocellatus* female (a

259   behaviour resembling oviposition but without insertion of the ovipositor into the mussel siphon

260   and without egg laying) and female *oviposition* (spawning, identified by a typical female

261   movement and insertion of her ovipositor into the mussel exhalant siphon). For a detailed

262   description of bitterling reproductive behaviour see Reichard et al. (2004b). *R. ocellatus*

263   behaviour was recorded continuously for 20 min. or until oviposition, whichever occurred

264   earlier. If oviposition occurred, the mussel was observed for a further 1 min. to record any egg

265   ejections by the host mussel. Mussel ejections occur either within a few seconds of oviposition

266   (immediate ejections) or over an extended period of several days (late ejections). The two types

267   of ejections are positively correlated (Reichard et al. 2007a, b) but late ejections cannot be

268   observed directly. After completion of a trial the experimental host mussels were replaced with

269   the next treatment combination and covered until the subsequent behavioural test, with a

270   minimum interval of 30 min. between tests to ensure that females were capable of spawning

271   another batch of ovulated eggs (Smith et el. 2004). Experimental mussels did not contain any

272   bitterling eggs or embryos prior to their use in a trial. Once all trials were completed for a given

273   pair of fish, they were stocked in a separate tank and not used again in the experiment.

274   **Data analysis**

275   Data from each focal female and male *R. ocellatus* were used to model host mussel preference.

276   Before applying statistical models a data exploration was undertaken, following the protocol

277   described in Ieno and Zuur (2015). The data were examined for outliers in the response and

278   explanatory variables, homogeneity and zero inflation in the response variable, collinearity

279   between explanatory variables and the nature of relationships between the response and

280   explanatory variables. Oviposition was used as a definitive measure of mussel preference. We

281   detected collinearity between female inspection of host mussels with oviposition and dropped

282   female inspection from the analysis. Male mussel inspection behaviour was similarly found to be

283   collinear with male leading behaviour and was subsequently dropped from the analysis. Male

284   leading behaviour was included in the model as a covariate to accommodate the effect of male

285   behaviour on female oviposition-site decision. Sperm release by males rarely occurred prior to

286   oviposition, limiting its informative value, and this variable was not included in the model. No

287   outliers were detected.

288   Mussel preference was modelled using a Bernoulli Generalized Linear Mixed Model

289   (GLMM). The model contained fixed effects for *sympatry* (two levels: sympatry and allopatry)*,*

290   *local coexistence* (two levels: locally coexisting and ecological naive), and *host species* (two

291   levels: *A. woodiana* and *U. douglasiae*). An interaction between host mussel species and local

292   coexistence was included to test for an effect of conditioning on host preference. *Male leading*

293   was a covariate representing the frequency of male leading behaviour to a mussel, which can

294   potentially influence female oviposition decisions (Smith et al. 2004). We also included the

295   effects of body size (measured as the total length, including caudal fin) of male and female *R.*

296   *ocellatus*. Mussel shell size was not included as a covariate because host mussels were size-

297   matched within host species and fully collinear (non-overlapping size range) between species.

298   Random terms were *Fish ID* (permitting random variation on the intercept for each individual

299   fish (fish pair used in trials) and *Trial* as a random intercept for each pairwise experimental trial

300   (with response to two host mussel individuals tested simultaneously in a paired design) nested

301   within individual fish and permitting a different intercept for each experimental trial.

302   The occurrence of egg ejection by mussels immediately following oviposition was

303   modelled using a subset of the data that included only trials that resulted in oviposition. The

304   model was fitted as a Bernoulli GLMM (log-link function) and contained the same set of fixed

305   and random effects as the oviposition model, except for the removal of *male leading* given the

306   lack of an association between male leading behaviour and host mussel ejection. Data analyses

307   were conducted using the *lme4* package in R 3.2.0 (Bates et al. 2014; R Core Development Team

308   2015). We quantified model goodness-of-fit (R^2_{GLMM}) for the fixed part of the model (R^2_{GLMM}

309   marginal) and for the complete model (including random effects; R^2_{GLMM} conditional)

310   (Nakagawa and Schielzeth 2013). Goodness-of-fit parameters were extracted using the *MuMIn*

311   package (Bartoń 2015). The *effects* package (Fox 2016) was used to visualize model estimates of

312   fixed term effects.

313

314   **Results**

315   A total of 272 paired trials were conducted, of which 80 resulted in oviposition. *Rhodeus*

316   *ocellatus* spawned more frequently in allopatric than sympatric mussel species, regardless of

317   mussel population of origin (Table 2a, Fig. 2), which is in agreement with a predominant role for

318   *host resistance* and contradicts predictions for the importance of *parasite specialization at the*

319   *meta level*. There was no effect of local coexistence between R. ocellatus and host mussels on the

320   probability of R. ocellatus oviposition, suggesting the parasite specialization was not affected by

321   local interactions and, therefore, not supporting predictions for *parasite specialization at the*

322   *local level*. There was no effect of host mussel species on the probability of R. ocellatus

323   oviposition, indicating that there was no parasite specialization for a particular host species.

324   There was no interaction between host mussel species and the effect of local coexistence,

325   indicating no effect of local conditioning on host preference. Male leading behaviour, a covariate

326   in the model, had a significant positive effect on the probability of oviposition (Table 2a). There

327   was no effect of female R. ocellatus body size on oviposition but a positive trend of male R.

328   *ocellatus* body size (Table 2a), indicating that females were more likely to spawn with larger

329   males. The model explained 9% of variability in the data (R^2_{GLMM} marginal = 0.0887), with

330   almost all explained variation related to fixed part of the model (R^2_{GLMM} conditional = 0.0887).

331   Ejection of *R. ocellatus* eggs by host mussels showed a significant effect of mussel

332   species (Table 2b), with *A. woodiana* exhibiting a higher frequency of egg ejection than *U.*

333   *douglasiae* (Fig. 3). Sympatric mussel hosts, irrespective of species, tended to eject *R. ocellatus*

334   eggs more frequently, though this effect was not statistically significant ($P = 0.068$). This trend

335   tends to support a role for *host resistance at the meta level*. There was no effect of local host

336   mussel coexistence on egg ejections (Table 2b), discounting the importance of local level effects.

337   The model explained 30% of variability in the data (R^2_{GLMM} marginal = 0.2997), with almost all

338   explained variation related to the fixed part of the model (R^2_{GLMM} conditional = 0.2996).

339

340   **Discussion**

341   We investigated outcomes in the host-parasite relationship between *R. ocellatus* and its unionid

342   mussel hosts with respect to parasite specialization and host resistance. Using an experimental

343   design that allowed us to distinguish the effects of host-parasite coexistence on a larger

344   geographical level (sympatry/allopatry) and local population level (ecologically relevant local

345   coexistence), we found support for the role of host resistance, with its effects operating at a

346   regional rather than local level. *R. ocellatus* avoided oviposition in sympatric populations of two

347   host mussel species in favour of allopatric populations and there was no effect of local host-

348   parasite coexistence or host species identity on *R. ocellatus* host preference. Ejection of *R.*

349   *ocellatus* eggs by host mussels was more likely by populations of one species, *A. woodiana*. This

350   host species has higher local population densities and a wider distribution than the other test host

351   species and is, therefore, more likely to be encountered by *R. ocellatus* and other bitterling

352   species. In addition, ejections tended to be higher in sympatric host mussel populations

353   irrespective of host species, providing further support for the importance of host resistance at a

354   meta level.

355   Parasite preference for allopatric hosts may interact with host species preference. We

356   found no difference in the preference of *R. ocellatus* between host mussel species, although *A.*

357   *woodiana* mussels exhibited overall higher egg ejection rates than *U. douglasiae* mussels (Table

358   2b). The lack of preference for host species may have been overridden by a strong preference for

359   allopatric hosts. *R. ocellatus* is a host generalist, capable of successfully parasitizing both mussel

360   species offered in the study, but demonstrating a certain level of host species preferences

361   (Reichard et al. 2007). Moreover, conditioning has been shown to affect the choice of host

362   mussel species in the European bitterling *R. amarus*, with a tendency to preferentially exploit

363   host mussel species that were housed with experimental fish (Reichard et al. 2011). In our study,

364   a population of *R. ocellatus* from Nanchang had no previous experience with *U. douglasiae*

365   hosts, but did not exhibit any mussel species preference towards *A. woodiana* hosts. This

366   outcome contradicts the potential role for a conditioning effect and reinforces the importance of

367   generalised host suitability (i.e. the lack of host resistance) in the oviposition-site decisions of *R.*

368   *ocellatus*. The cues that bitterling use for oviposition decisions appear to include mussel odour,

369   the dissolved oxygen concentration of the water emerging from the mussel and the flow velocity

370   of the exhalant flow (Smith et al. 2001; Mills and Reynolds 2002; Phillips et al. 2017), though

371   these are not yet fully understood. In European unionids, host mussel populations have evolved

372   morphological and behavioural adaptations, including egg ejection, to avoid bitterling

373   oviposition, with mussel populations from areas under stronger parasitic pressure exhibiting

374   enhanced counter-adaptations to reduce parasitism by bitterling (Reichard et al. 2010). It is

375   unclear whether bitterling are capable of perceiving such differences in the level of host counter-

376   adaptation, but our results suggest it may be the case at the host population level, making *R.*

377   *ocellatus* highly efficient at utilising a broad range of novel host populations and species.

378   However, despite showing appropriate responses, the cues used by *R. ocellatus* to recognise host

379   suitability remain to be identified.

380   The limited species selectivity by *R. ocellatus* may appear contradictory in the context of

381   host-parasite coevolution, but may reflect an overriding role for host quality traits rather than

382   host specific traits. Thus the dissolved oxygen concentration and flow velocity of the exhalant

383   flow of a mussel may be more reliable predictors of the suitability of a host for the incubation of

384   eggs and embryos than species identity, at least in the case of *R. ocellatus*. This effect arises

385   because host quality, irrespective of species, can change markedly over a spawning season

386   (Kitamura 2005; Smith 2017). The spawning season of *R. ocellatus* is relatively protracted,

387   starting in April and typically lasting 6 months (Kitamura 2005; Pateman-Jones et al. 2011). As

388   the spawning season advances mussels fill with eggs and embryos and progressively decline in

389   quality as incubation sites. Towards the end of the spawning season bitterling offspring emerge

390   from mussels, which consequently increase in quality as hosts (Kitamura 2005). While we have

391   only tested host mussels without bitterling eggs and embryos to standardise host condition, the

392   capacity to distinguish the relative quality of an individual mussel when making oviposition-site

393   decisions may be more critical than host species discrimination. Additional studies to compare

394   egg ejection rates among more host species and hosts with varying intensity of infection by *R.*

395   *ocellatus* would help clarify variation in host preference.

396   The host mussel choice by *R. ocellatus* was adaptive in terms of the use of host mussel

397   populations that tended to be least effective in ejecting *R. ocellatus* eggs. We have previously

398   detected large differences in capability of European unionid hosts to eject the eggs of the

399   European bitterling, *R. amarus*, between areas of recent and ancient association (Reichard et al.

400   2010). However, cross resistance experiments between host mussel and fish parasite populations

401   from areas of ancient and recent sympatry were not possible in that study due to the lack of legal

402   approval of animal transport between study regions. The current findings with *R. ocellatus* and

403   their host mussels are consistent with the prediction that naive hosts, evolving in the absence of

404   parasites, relax costly resistance mechanisms in comparison with hosts continuously exposed to

405   parasites. Similarly, relaxed selection by brood parasitic brown-headed cowbirds *Molothrus ater*

406 (Boddaert) on allopatric populations of American robins *Turdus migratorius* L. and gray catbirds

407 *Dumetella carolinensis* (L.) resulted in a lower frequency of ejection of parasite eggs compared

408 with sympatric populations (Kuehn 2009). Analogous observations have been found in other

409 host-parasite systems, such as for the susceptibility of isopod populations to an acanthocephalan

410 parasite (Hasu et al. 2009), and infection by the yeast parasite *Metschnikowia bicuspidata*

411 (Metschnikow) of the crustacean *Daphnia dentifera* Forbes (Auld et al. 2013).

412 No effect of local coexistence on host mussel preference across *R. ocellatus* populations

413 failed to support the existence of fine-scale local adaptations of *R. ocellatus* to avoid their locally

414 coexisting population of hosts. Parasites are frequently reported to avoid local hosts due to their

415 better resistance, for example in the relationship between the bacterial parasite *Holospora*

416 *undulata* Hafkine and its protozoan host *Paramecium caudatum* Ehrenberg (Adiba et al. 2010).

417 However, the lack of host-parasite coevolution at a local scale could be explained by other

418 factors. First, studies based on parasites with broad host ranges are less likely to demonstrate

419 local parasite adaptation than those on parasites with narrow host ranges, probably due to

420 evolutionary lags during diffuse coevolution of generalist parasites with their hosts (Lajeunesse

421 and Forbes 2002). In terms of our study system, this would predict more localised coevolutionary

422 dynamics in host specialist bitterling, such as *R. sinensis* (Reichard et al. 2007a). Second, the

423 potential for large-scale dispersal by at least one of the partners mitigates the opportunity of fine-

424 scaled local adaptation. All unionid mussels have a larval stage (glochidium) that attaches to host

425 fish for days to months and may be dispersed over large distances (Dillon 2000). Third, in some

426 systems, local adaptation of the parasite can be observed over a scale of meters (Lively and

427 Jokela 1996), while in other systems parasites exhibit no local adaptation within-regions but do

428 express adaptations at larger distances (Hanks and Denno 1994). Hence, although it is possible

429   that *R. ocellatus* exhibit no local adaptation at the scale tested here, we cannot preclude local

430   adaptation on a larger environmental scale, particularly given the limited number of test

431   populations and the use of naive mussels as one of the host populations.

432   Understanding the coevolutionary dynamics between *R. ocellatus* and *A. woodiana* has

433   become a pressing question because *A. woodiana* is an invasive species across Europe, Asia, and

434   the Americas (Watters 1997; Douda et al. 2012). The invasive range of *A. woodiana* includes

435   large parts of the range of the European bitterling *R. amarus* (Reichard et al. 2015). While we

436   show that *A. woodiana* was capable of ejecting a large proportion of eggs oviposited by *R.*

437   *ocellatus* in its gills, its ejection rate of *R. amarus* eggs is even greater (Reichard et al. 2012;

438   Reichard et al. 2015). Notably, *R. ocellatus* is itself an invasive species in several Asian

439   countries (Welcomme 1988; Kawamura et al. 2001; Vasil'eva et al. 2015). Our results show a

440   preference for allopatric, evolutionarily naive hosts, regardless of host species identity. In order

441   to establish, persist and spread without their native hosts, introduced parasites must either be

442   generalist species that can infect a large range of new hosts, with at least some degree of success,

443   or specialize on a new host species in the novel communities they invade (Font 2003; Frankel et

444   al. 2015). One of the primary reasons for the invasive success of *R. ocellatus* may be its capacity

445   to exploit a wide variety of mussel species and populations as hosts, coupled with the fitness

446   advantage demonstrated here in using naive host populations due to their lower egg ejection rates

447   (Reichard et al. 2010). Given that several successful parasite invasions have been attributed to

448   the use of naive hosts species owing to the absence of an effective adaptive response (Kelehear et

449   al. 2015), *R. ocellatus* is predicted to successfully reproduce and establish non-native populations

450   in other geographic regions.

451

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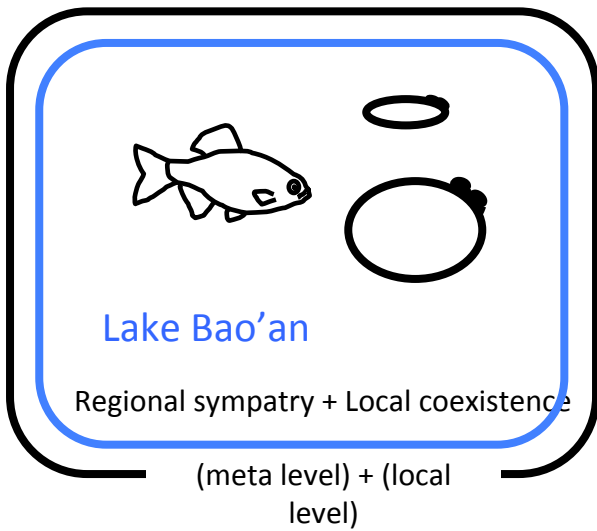612   577

Figure Captions

Figure 1 An illustration of experimental populations with regard to their reciprocal coexistence at a meta-level (sympatry/allopatry) and local coexistence (locally coexisting/ecologically naïve). Each experimental pair of *Rhodeus ocellatus* was tested against four contrasting pairs of host mussels, with all four trials completed over 1-3 days. The trials represented contrasts between (1) *Anodonta woodiana* from two populations; and (2) *Unio douglasiae* from two populations, each contrasting geographically sympatric and allopatric hosts; (3) *A. woodiana* and *U. douglasiae* from Lake Bao'an, contrasting host species that both locally coexisted with *R. ocellatus*; and (4) *A. woodiana* and *U. douglasiae* from Lake Qinglan, contrasting host species that were both ecologically naïve to *R. ocellatus*. Note that since two *R. ocellatus* populations were used overall, each contrast had additional predictions that are articulated in Table 1.

Figure 2 Mean rate of oviposition of female *R. ocellatus* with respect to (a) sympatry/allopatry between *R. ocellatus* (parasite) and host mussel populations, (b) local coexistence between *R. ocellatus* (parasite) and host mussel populations, (c) host mussel species regardless their sympatry or local coexistence with *R. ocellatus*. Values represent mean and 95% confidence intervals from model estimates (Table 2a), produced in the R package *effects*. An asterisk indicates $P < 0.05$.

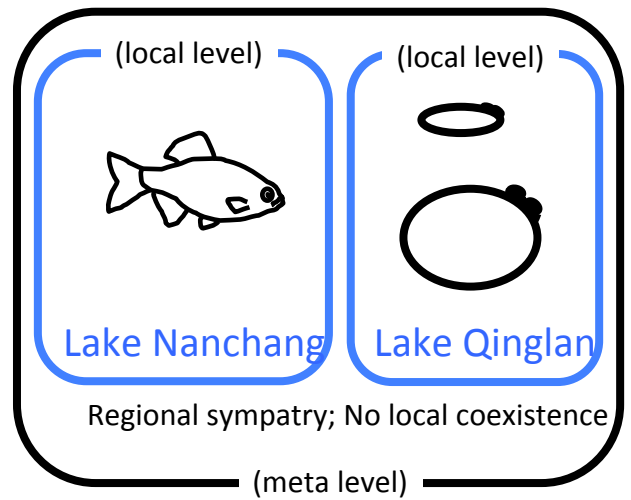**Figure 3** Mean rate of *R. ocellatus* egg ejection by host mussels with respect to (a) sympatry/allopatry between *R. ocellatus* (parasite) and host mussel populations; (b) local coexistence between *R. ocellatus* (parasite) and host mussel populations; (c) host mussel species regardless their sympatry or local coexistence with *R. ocellatus*. Values represent mean and 95%
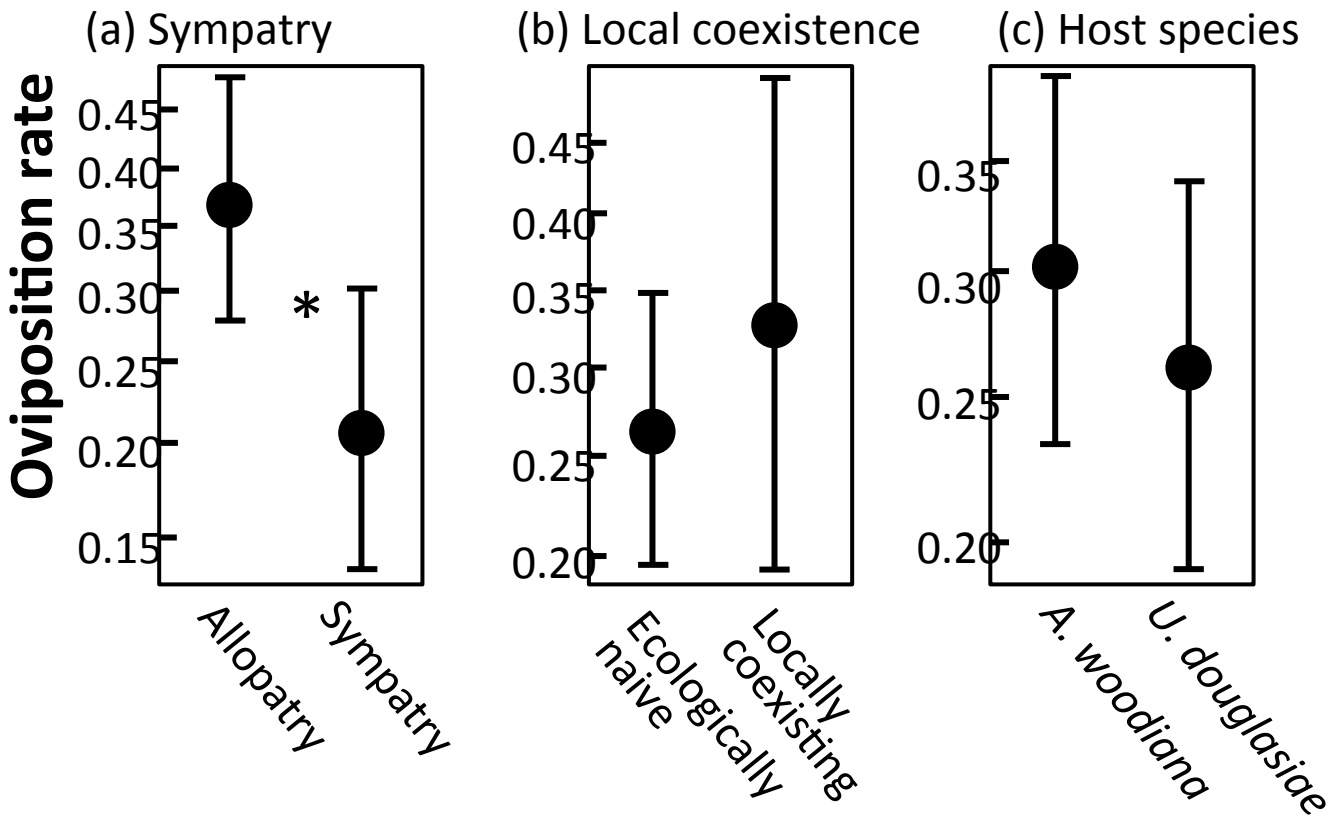
636   confidence intervals from model estimates (Table 2b), produced in the R package *effects*. An

637   asterisk indicate $P < 0.05$, asterisk in parentheses indicates $P = <0.10$.

Hubei Province

Lake Bao'an

Regional sympatry + Local coexistence

(meta level) + (local level)

Jianxi Province

(local level)   (local level)

Lake Nanchang   Lake Qinglan

Regional sympatry; No local coexistence

(meta level)

(a) Sympatry (b) Local coexistence (c) Host species

(a) Sympatry　(b) Local coexistence　(c) Host species

Ejection rate

(a) Sympatry
- 0.8
- 0.7
- 0.6
- 0.5
- 0.4
- 0.3
- 0.2

(*)

Allopatry　Sympatry

(b) Local coexistence
- 0.7
- 0.6
- 0.5
- 0.4
- 0.3
- 0.2
- 0.1

Ecologically naive　Localy coexisting

(c) Host species
- 0.8
- 0.7
- 0.6
- 0.5
- 0.4
- 0.3
- 0.2
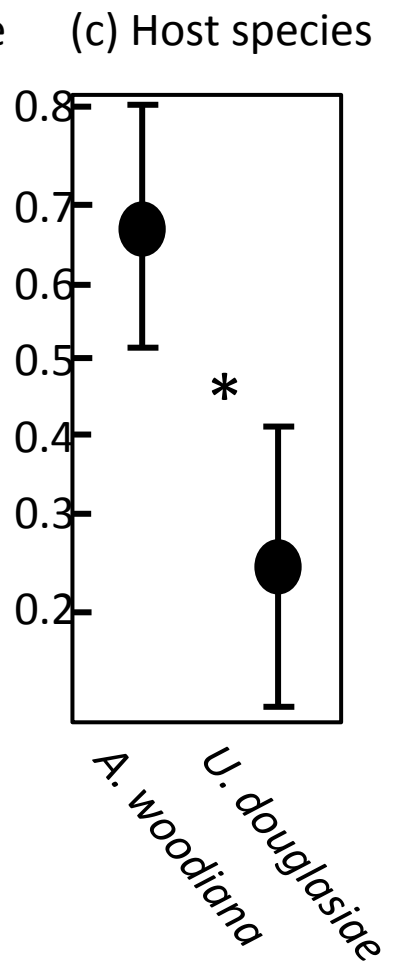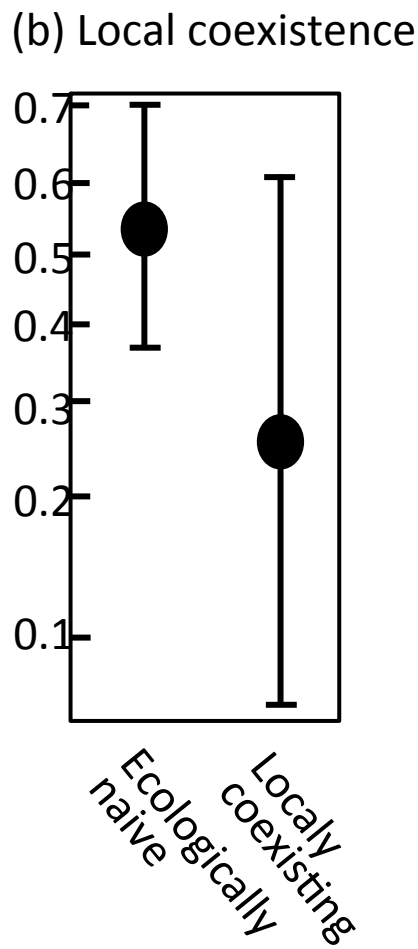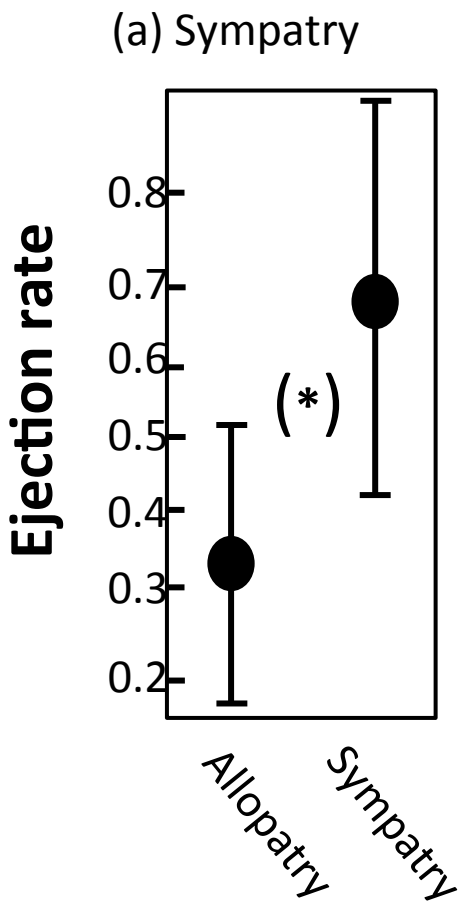
*

A. woodiana　U. douglasiae

Table 1. Experimental design showing the predicted responses of parasitic *R. ocellatus* and host mussels for scenarios when parasite specialisation (tested using parasite oviposition preference) or host resistance (tested using host egg ejection) dominate the current coevolutionary state. Support for each contrast is indicated by an asterisk (at *P* < 0.05) or asterisk in brackets (at *P* = < 0.10).

| Response variable | Covariate | Covariate level | Predictions | | Support |
			Parasite specialisation	Host resistance	
Parasite oviposition	Sympatry	Sympatric	✔	✗	∗
		Allopatric	✗	✔	
Parasite oviposition	Local coexistence	Coexisting	✔	✗	–
		Not coexisting	✗	✔	
Host egg ejection	Sympatry	Sympatric	✗	✔	∗
		Allopatric	✔	✗	
Host egg ejection	Local coexistence	Coexisting	✗	✔	–
		Not coexisting	✔	✗	

✔ – preference for this host supports the prediction

✗ – preference for this host contradicts the prediction

Table 2. Estimates and *P*-values of fixed effects from GLMMs on (a) oviposition rate; and(b) egg ejections. Significant *P*-values are indicated in bold.

Variable	Source	Estimate	S.E.	*z*	*P*
(a) Oviposition	Intercept	**-0.528**	**0.241**	**-2.19**	**0.028**
	Sympatry(sympatric)	**-0.834**	**0.388**	**-2.15**	**0.032**
	Local coexistence(coexisting)	0.458	0.544	0.84	0.401
	Sympatry : Local coexistence	-0.370	0.633	-0.58	0.559
	Mussel species(U.douglasiae)	-0.135	0.319	-0.42	0.673
	Male leading	**0.364**	**0.137**	**2.66**	**0.008**
	Female *R. ocellatus* size	-0.115	0.152	-0.76	0.447
	Male *R. ocellatus* size	0.262	0.143	1.83	0.067
(b) Egg ejection	Intercept	0.490	0.388	1.26	0.207
	Sympatry(sympatric)	1.509	0.823	1.83	0.067
	Local coexistence(coexisting)	-1.230	0.966	-1.27	0.203
	Mussel species(U.douglasiae)	**-1.872**	**0.529**	**-3.54**	**<0.001**
	Female *R. ocellatus* size	0.306	0.288	1.06	0.288
	Male *R. ocellatus* size	-0.281	0.262	-1.07	0.283