1 # The role of local adaptation in shaping fish-mussel coevolution

2 Running title: Freshwater mussel-host coadaptation

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4 Karel Douda[1*], Huan-Zhang Liu[2], Dan Yu[2], Romain Rouchet[3], Fei Liu[2], Qiong-Ying Tang[2], Caroline

5 Methling[3,4], Carl Smith[3,5,6], Martin Reichard[3]

6

7 [1] Czech University of Life Sciences Prague, Department of Zoology and Fisheries, Kamýcká 129, Prague, CZ 165

8 00, Czech Republic

9 [2] The Key Lab of Aquatic Biodiversity and Conservation, Institute of Hydrobiology, Chinese Academy of Sciences,

10 Wuhan 430072, Hubei, China

11 [3] Institute of Vertebrate Biology, Academy of Sciences of the Czech Republic, Květná 8, Brno, CZ 603 65, Czech

12 Republic

13 [4] Technical University of Denmark, Kongens Lyngby, Copenhagen, Denmark

14 [5] School of Biology and Bell-Pettigrew Museum of Natural History, University of St Andrews, St Andrews KY16

15 8LB, United Kingdom

16 [6] Department of Ecology & Vertebrate Zoology, University of Łodz, Banacha 12/16, 90-237 Łodz, Poland

17

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19 Correspondence

20 Karel Douda, Czech University of Life Sciences Prague, Department of Zoology and Fisheries, Kamýcká 129,

21 Prague, CZ 165 00, Czech Republic. E-mail: k.douda@gmail.com

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26 **Abstract**

27 1. The survival of affiliate (dependent) species in a changing environment is determined by the

28   interactions between the affiliate species and their available hosts. However, the patterns of

29   spatial and temporal changes in host compatibility are often unknown despite host shifts

30   having direct impact on the persistence of local populations. Bivalves of the order Unionida

31   (freshwater mussels) are a functionally important but declining group of affiliate species, which

32   are dependent on freshwater fish to host their parasitic larvae. The role of local adaptations

33   and host fish resistance in shaping freshwater mussel host relationships remains poorly

34   understood.

35 2. We used an invasive East Asian bivalve, *Sinanodonta woodiana* (Lea 1834), and its potential

36   host fishes to study the mechanisms shaping fish-mussel coevolution using a combination of

37   laboratory cross-exposure methods and field-collected data. We tested whether generalist

38   host use of *S. woodiana* is pertinent to native host species, with special attention to bitterling

39   fishes (Acheilognathinae) that are characterized by a mutual association with freshwater

40   mussels. We also tested whether the pattern of the parasite-host association varies temporally

41   (between areas of ancient and recent sympatry) and spatially (at a sub-basin level in its native

42   range).

43 3. Results revealed the ability of *S. woodiana* to widely exploit non-bitterling host fishes at a

44   global scale. In contrast, the ability of *S. woodiana* to exploit closely associated bitterling

45   fishes was low in its native range (with ancient sympatry). In areas of recent sympatry (non-

46   native *S. woodiana* range in Europe), *S. woodiana* glochidia were demonstrated to readily

47   parasitize local, evolutionarily naive bitterling species at high density.

48 4. The results of a population-level experiment with three native populations of *S. woodiana* and

49   rose bitterling, *Rhodeus ocellatus*, from various sub-basins of the River Yangtze confirmed

50   that mussel populations vary in their compatibility with particular host populations. However,

51   there was no evidence of population-specific adaptive coevolution.

52 5. This study provides the first evidence for a role of fish counter-adaptations against freshwater

53   mussel glochidia, and documents the importance of population-level variation in shaping

54   compatibility between glochidia and their host fishes. This outcome can inform predictions on

55   the impact of biotic homogenization on endangered affiliate species in general and freshwater

56   mussels in particular.

57   **1 | INTRODUCTION**

58   The existence of many functionally important taxa depends on vulnerable links among interacting

59   species within communities (Koh et al., 2004; Moir et al., 2011). Increasing anthropogenic pressure on

60   freshwater habitats pushes many dependent (affiliate) species to interact with a novel set of potential

61   hosts which may limit their persistence (Vaughn, 2012). A substantial research effort has focused on

62   the consequences of species loss in host-affiliate networks and resulting co-extinction risk (Campião,

63   Ribas, Cornell, Begon, & Tavarez, 2015; Spooner, Xenopoulos, Schneider, & Woolnough, 2011;). This

64   approach has significantly improved our understanding of the risk of imminent impacts, but our

65   knowledge of the potential role of host switching and affiliate-host coevolution remains limited for most

66   affiliate species lineages (Colwell, Dunn, & Harris, 2012).

67   One of the most characteristic examples of affiliate species in the freshwater environment are

68   freshwater mussels of the order Unionida (Bivalvia). These benthic invertebrates are a functionally

69   important component of freshwater ecosystems (Vaughn & Hakenkamp, 2001) and their extirpation

70   can lead to long-term losses in mussel-provided ecosystem services (Vaughn, Atkinson, & Julian,

71   2015). Unionid bivalves are typically dependent on a limited range of species or lineages of freshwater

72   fish (Barnhart, Haag, & Roston, 2008), which are obligatory hosts of their short-term parasitic larval

73   stage (glochidium). Glochidia are not capable of active host selection when released from a parent

74   mussel and die within a few days if they attach to an incompatible host or do not attach at all (Kat,

75   1984). The lack of compatible hosts in mussel habitats can limit species dispersal (Schwalb, Cottenie,

76   Poos, & Ackerman, 2011), but can also be the critical factor in the decline of many mussel species

77   (Lopes-Lima et al., 2014; Lydeard et al., 2004). While recognition of the importance of host resources

78   for the persistence of mussels is increasing, little is known about the mechanisms of adaptations to

79   host fishes and the evolutionary potential of mussels to adapt to a shifting host community.

80   In this coevolutionary relationship, the strength of selection on mussels and fish, respectively, is

81   expected to be unbalanced (Barnhart, Haag, & Roston, 2008; Bauer, 1997). Mussels are predicted to

82   experience strong selection on host-compatible genotypes in each generation as the survival of

83   mussel larvae critically depends on host compatibility. In contrast, selection on resistance to glochidia

84   in fish genotypes is likely weaker because mussels are only short-term parasites that usually occupy

85   only a fraction of its host's range and parasitize only a limited proportion of the host population

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86   (Barnhart, Haag, & Roston, 2008; Nuismer, Thompson, & Gomulkiewicz, 2003). As a result, host

87   suitability will be primarily determined by adaptations of mussels to a specific host fish species.

88   However, despite an usually low prevalence and abundance of glochidia in fish populations (Blažek &

89   Gelnar, 2006; Martel & Lauzon-Guay, 2005), recent findings indicate that glochidia can have multiple

90   effects on host fish physiology (Ooue, Terui, Urabe, & Nakamura, 2017; Slavík et al., 2017), behavior

91   (Filipsson et al., 2016; Horký, Douda, Maciak, Závorka, & Slavík, 2014) and condition status (Douda et

92   al., 2016), which can operate at environmentally relevant glochidia densities. These potentially

93   substantial costs to fish of hosting glochidia could play a significant role in mussel-fish coevolution.

94   An emerging question regards the geometry of local adaptations and host-parasite coevolution

95   between the mussels and fish. Specifically, the coevolution of physiological compatibility may be

96   shaped primarily by mussel adaptations against a non-specific fish immune response, leading to a

97   situation whereby mussels are best adapted to exploit the most available and ecologically compatible

98   host species (*encounter selection hypothesis*). Alternatively, fish may be selected to avoid parasitism

99   by glochidia and evolve resistance to glochidia infection, with the most exposed and ecologically

100   compatible hosts showing greatest resistance. Despite an increase in the understanding of parasite

101   local adaptations (reviewed by Greischar & Koskella, 2007), data on glochidia-fish relationships are

102   limited, and our understanding of host specialisation by freshwater mussels remains opaque.

103   In this study, we use *Sinanodonta woodiana* (Lea 1834), a unionid mussel with a large natural

104   distribution in Asia (He & Zhuang, 2013) and invasive elsewhere (e.g. in Europe and Indonesia)

105   (Bolotov et al., 2016; Watters, 1997), using a wide range of potential host fishes. Glochidia of *S.

106   woodiana* are relatively large (390-400 μm), triangular in shape and attach to fish fins and gills where

107   they became encysted, parasitizing the host fish for several days depending on ambient temperature

108   (Dudgeon & Morton, 1984). It has recently been documented that *S. woodiana* can cause significant

109   harm to its hosts (Douda et al., 2016; Slavík et al., 2017), supporting the possibility that potential host

110   species might evolve resistance against *S. woodiana* glochidia. From data collected in the non-native

111   part of its range, *S. woodiana* is considered to be a generalist host whose larvae are capable of

112   development on almost all available fish species (Douda, Vrtílek, Slavík, & Reichard, 2012; Watters,

113   1997). Data collected in its native range, in contrast, illustrate that there are differences in host quality

114   (Dudgeon & Morton, 1984; Hua, Xu & Wen, 2005). Moreover, data from non-native *S. woodiana*

115   indicate population-specific variability in host compatibility with one host species, the European

116   bitterling (*Rhodeus amarus*), corresponding with the contrasting histories of recent sympatry between

117   *R. amarus* and *S. woodiana* at the population level (Reichard et al., 2015).

118   Bitterling fishes are in a unique position with respect to their association with freshwater mussels.

119   Bitterling (Acheilognathinae: Cyprinidae) parasitize freshwater mussels by laying their eggs in the

120   mussel gill cavity where bitterling eggs and embryos complete development over a period of

121   approximately 4 weeks (Smith, Reichard, Jurajda, & Przybylski, 2004). All bitterling, therefore, spend

122   some part of their lives in close association with adult mussels. Notably, the bitterling mating system

123   involves males guarding territories around freshwater mussels where reproductively active females are

124   courted over an extended reproductive period (2-6 months). The spawning behaviour of male and

125   female bitterling involves frequent intimate association with adult mussels, such as regular inspection

126   of mussel siphons (Phillips, Reichard, & Smith, 2017; Spence, Reichard, & Smith, 2013). Such

127   behavior makes bitterling potentially vulnerable to glochidial infection, particularly as mussels expel

128   glochidia during late spring and early summer at the height of the bitterling spawning season.

129   The aim of this study was to test whether *S. woodiana* functions as a host generalist in both the native

130   and non-native part of its range, using a combination of laboratory experiments and data from natural

131   populations. By comparing infection patterns in the native and non-native range of *S. woodiana* we

132   were able to investigate whether the outcome of parasite-host conflict varied between the areas of

133   ancient sympatry and recently invaded area (long-term temporal axis) and over a spatial scale (at a

134   sub-basin level in the native range). The specific aims of the study were to: 1) experimentally compare

135   the physiological compatibility of *S. woodiana* glochidia for infecting bitterling and non-bitterling fish

136   between a native and non-native range; 2) experimentally evaluate the degree of population-level

137   variation in host compatibility in *S. woodiana*'s native range and relate to the results for its non-native

138   range; 3) quantify the natural levels of *S. woodiana* glochidia infection of fishes in its native and non-

139   native range and thus; 4) evaluate the implications for the evolution of host relationships in freshwater

140   mussels.

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142   **2 | METHODS**

143   2.1 | Species level host compatibility of *S. woodiana*

144   Study populations were located in the River Yangtze Basin (Fig. 1), which is considered to be

145   the likely site of origin of invasive European populations (Bolotov et al., 2016). This is also the area of

146   ancient sympatry between *S. woodiana* and several bitterling species (Reichard, Liu, & Smith, 2007).

147   Gravid females were collected in May and June 2014 from Lake Bao'an (population B, Hubei province,

148   China, 30°17'25.4"N 114°43'48.9"E). Female mussels were transported to the laboratory and stored in

149   1350 L shallow containers with aerated tap water. Ripeness of mussel glochidia was regularly checked

150   by visual inspection of marsupium colour (a dark purple colour indicates advanced developmental

151   stage, K. Douda, personal observation). Ripe glochidia were obtained by flushing the marsupium with

152   water from a syringe. The viability of glochidia (a subset of 30 individuals for each mussel) was verified

153   by evaluation of their snapping action in a sodium chloride solution. Glochidia from three gravid

154   females with a viability exceeding 90% were pooled and used for inoculation. Nine potential host

155   species (Table 1) from four fish families (Cyprinidae, Siluridae, Gobiidae, Cobitidae) were used for

156   experimental infection by glochidia. *Carassius auratus, Rhinogobius giurinus, Hypophthalmichthys*

157   *nobilis, Sinibotia superciliaris, Silurus meridionalis, Plagiognathops microlepis* are native Chinese fish

158   species with extensive range overlap with *S. woodiana.* As a control, we also included *Danio rerio,* a

159   species of Indian origin (Spence, Gerlach, Lawrence, & Smith, 2008). Two common and widely

160   distributed Asian bitterling species, *R. ocellatus* and *R. sinensis*, were tested as hosts with a

161   persistently high risk of parasitism. Experimental fish were obtained from commercial hatcheries or

162   rearing facilities of the Institute of Hydrobiology of the Chinese Academy of Science in Wuhan, except

163   for *R. giurinus* and both *Rhodeus* species which were collected using dip nets from Lake Bao'an.

164   Seven to twenty-six individual fish per species were used for infections; a total of 105 individually

165   monitored fish (Table 1). Experimental fish were either in the first or second year of life. Six fish (four

166   *H. nobilis* and two *C. auratus*) died before the end of juvenile detachment (mortality was not related to

167   experimental infection) and were not included in the analysis.

168   Fish were infected following the protocol of Douda, Vrtílek, Slavík and Reichard (2012) by placement

169   into a common bath suspension (one infection tank for all species, total volume 50 L) of dechlorinated

170   tap water and containing 3860 ± 2097 (mean ± s.d.) viable glochidia L^{-1}. The glochidia were kept in

171   homogeneous suspension through aeration. After 15 min of inoculation, fish were transferred into an

172   aerated bath that did not contain glochidia for 30 min to rinse off non-attached glochidia. Fish were

173   then individually placed into 105 continuously aerated 5-L plastic tanks with dechlorinated tap water

174   that had nets with 3 mm mesh size on the bottom. Fish were fed daily with a commercial flake fish

175   food. The temperature in the tanks was 24.9 ± 0.5 °C (mean ± s.d.) during the experiment. Water in

176   individual tanks was filtered through nylon screens (mesh size 139 µm; UHELON) and partially

177   exchanged (80% of water volume) every second day for 14 days in total. Untransformed glochidia and

178   juvenile mussels were washed from the screens, isolated from debris and counted under a

179   stereomicroscope. All individuals that were collected were inspected; if foot activity or valve movement

180   were observed, individuals were classified as live juveniles.

181   In order to compare experimental data on physiological host compatibility between the native and non-

182   native range of *S. woodiana* we used the results of host compatibility testing by Douda, Vrtílek, Slavík

183   and Reichard (2012) as a comparative dataset. Data on the metamorphosis success rate of *S.*

184   *woodiana* and eight common fish species in Central Europe (family Cyprinidae, Supl. Table S1),

185   including one bitterling species (*Rhodeus amarus*), were compared with experimental data from the

186   native range of *S. woodiana* collected in the present study. The European dataset included

187   metamorphosis success from six to ten individuals per fish species (age 0+, 1+) infested

188   experimentally with *S. woodiana* glochidia from the River Kyjovka (Danube basin) where the mussel

189   has been introduced (see Douda, Vrtílek, Slavík, & Reichard, 2012 for further details).

190   2.2 | Population level host compatibility of *S. woodiana*

191   Gravid females of *S. woodiana* were collected in May - June 2014 from three different populations in

192   the River Yangtze Basin, China (Fig. 1). Mussels syntopic with the experimental *R. ocellatus*

193   population were collected from Lake Bao'an (population B). Mussels syntopic with their local (non-

194   experimental) *R. ocellatus* population were collected from earthen fishponds near Jianli (population A,

195   Hubei province, 29°58'37.5"N, 112°42'29.4"E). Mussels naïve to *R. ocellatus* were collected from Lake

196   Qinglan (population C, Jiangxi province, 28°30'40.144"N, 116°8'2.112"E). The study populations of *S.*

197   *woodiana* were 187-353 km apart and were located in separated watersheds of three distinct

198   tributaries of the River Yangtze.

199   The majority of host fishes used in the study (syntopic *R. ocellatus, R. sinensis, R. giurinus*) were

200   collected at one of the mussel collection sites (population B, Bao'an Lake) in April and May 2014 using

201   baited fish traps. The second population of *R. ocellatus* (naïve to experimental populations of mussels)

202   was collected in a natural pond in the city of Nanchang (Jiangxi province, 28°39'55.08"N,

203   115°48'57.79"E), located 35 km from Lake Qinglan (mussel population C collection site). All fish were

204   transported to the laboratory in aerated boxes at least three weeks before the experiments to ensure

205   that no glochidia were attached to experimental fish. Artificially reared *Gobiocypris rarus* Ye & Fu,

206   1983, a cyprinid fish species from the native range of *S. woodiana* with an endemic and highly

207   restricted distribution, was obtained from a local rearing facility (Institute of Hydrobiology, Chinese

208   Academy of Science) and included in the analysis as a control species. The mean ± s.d. standard

209   length and mean ± s.d. wet body mass of experimental fish are reported in Table S2.

210   We used larvae from one parent mussel to simultaneously infect two to four individuals from each

211   tested fish strain (*R. ocellatus* – Lake Bao'an, *R. ocellatus* – Lake Qinglan, *R. sinensis*, *G. rarus*, *R.

212   giurinus*). Altogether, we performed 23 experimental infections (7-8 female mussels per each of the

213   three tested populations) to infect a total of 220 individual fish (14–16 individuals for each fish strain–

214   *S. woodiana* population combination). The fish were infected in aerated suspensions (0.5 L per fish)

215   with mean larval densities of 2885 ± 641 (mean ± s.d.) viable glochidia L^{-1}. A common infection bath

216   suspension was used in each inoculation, which was subdivided into five sections to prevent mixing of

217   fish individuals from different source populations. After a 15-min exposure, fish were transferred to

218   clean water for 30 min to rinse non-attached glochidia.

219   Fish were subsequently placed singly into continuously aerated 5 L plastic tanks (i.e. a total of 220

220   tanks). An initial host response was monitored using the same methods as for the species-level

221   experiment. Rejected larvae were collected from water using filters and counted 48 hours after

222   infection. The number of total attached glochidia was estimated by host dissection and direct

223   enumeration of remaining glochidia on the body of each fish. Mean water temperature was 25.0 ± 0.8

224   °C (mean ± s.d.) during the experiment. These methods enabled us to estimate both the absolute

225   number of attached larvae and the proportion of larvae that successfully survived the initial host

226   response. No fish died during the experiment.

227   We used the ratio between the number of initially attached glochidia per fish and the proportion of

228   glochidia that remained attached 48 hours after infection as a measure of glochidia–fish physiological

229   compatibility, hereafter referred to as 'infection success'. This parameter was used because the initial

230   host response represents a critical stage in the parasitic phase of unionid bivalve larvae (Dodd,

231   Barnhart, Rogers-Lowery, Fobian, & Dimock, 2005; Rogers-Lowery & Dimock, 2006). Additionally, it

232   has been shown that the shedding of immature glochidia in the initial period after infestation closely

233   corresponds to the overall success of glochidia parasitism (Douda, Horký, & Bílý, 2012). The absolute

234   number of attached glochidia per gram of fish body weight was also calculated for the experimental

235   fish and compared among treatments in order to compare relative susceptibility of fish strains to

236   glochidia attachment.

237

238   2.3 | Natural prevalence and abundance of *S. woodiana* glochidia on host fish

239   In order to complement physiological data with an understanding of ecological host compatibility in *S.*

240   *woodiana* we also quantified natural encystment rates. We quantified the prevalence and intensity of

241   infection of *S. woodiana* glochidia on fish collected in the field during the glochidia discharge season in

242   both the native Asian (Lake Bao'an) and non-native European range (River Kyjovka). Sites with the

243   occurrence of bitterling species were selected to enable the comparison of relative use of bitterling

244   species as hosts.

245   Fish were collected from the River Kyjovka using a standard electrofishing method (pulsed DC, 50 Hz,

246   Lena, Czech Republic) between July 10th 2013 and August 22nd 2015. Electrofishing has no impact on

247   glochidial attachment (Holliman, Kwak, Cope, & Levine, 2007). The River Kyjovka (Czech Republic) is

248   a small lowland river (4-6 m wide, <1 m deep) typical of the region. In Lake Bao'an, fish were collected

249   between May 23rd and June 12th 2014 using baited fish traps, which was the most effective method for

250   collecting small-sized fish at the site.

251   At both sites sampled fish were killed with an overdose of anaesthetic (MS-222) and immediately

252   placed in a fixative (70% ethanol, 4% formaldehyde) and transported to the laboratory. Glochidia of *S.*

253   *woodiana* were identified on the basis of characteristic morphology and counted under a dissection

254   stereomicroscope.

255

256 2.4 | Statistical analysis

257 Glochidia metamorphosis success on different host species (proportion of glochidia that successfully

258 transformed into juvenile stage) was compared among host species using generalized linear models

259 (GLM, logit link function and a quasi-binomial error structure). The dependent variable (metamorphosis

260 success) was treated as binary. Multiple comparisons were performed by Tukey's post-hoc test using

261 the *glht* function in the R package *multcomp* (Hothorn, Bretz, & Westfall, 2008) to examine pairwise

262 differences among host species.

263 Data on infection success of glochidia (proportion of glochidia that remained attached after 48 hours)

264 on different fish strain by population combinations were analysed in a generalized linear mixed model

265 framework (GLMM, logit link function and quasi-binomial error structure, *glmer* procedure), and data

266 on initially attached glochidia per gram in a linear mixed model framework (LMM, *lmer* procedure) in

267 the R package *lme4* (Bates, Maechler, Bolker, & Walker, 2015). The dependent variable (infection

268 success) was treated as a binary variable. The fixed effects included *Mussel population* (populations A

269 - Jianli, B - Bao'an, C - Qinglan), *Fish strain* (sympatric *R. ocellatus,* allopatric *R. ocellatus, R.*

270 *sinensis, R. giurinus, G. rarus*) and their interaction *Mussel population x Fish strain*. Random effects

271 (intercepts) included *Mussel individual* (infestation event) and *Host fish individual* (not included in the

272 model on attached glochidia per gram).

273 The comparison of *S. woodiana* glochidia natural infestation rates (number of recovered glochidia) on

274 bitterling and non-bitterling fish between native and non-native range sites was performed using

275 Fisher's Exact Test for Count Data (Sokal & Rohlf, 1995). All analyses were performed using the R

276 3.3.2 software package (R Core Development Team, 2016).

277

278 **3 | RESULTS**

279 3.1 | *Sinanodonta woodiana* host compatibility at the species level

280 Glochidia metamorphosis success rate differed significantly among host fish species (Fig. 2; GLM:

281 $F_{10,142} = 17.0$, $p < 0.001$). Mean metamorphosis success rate of *S. woodiana* on Asian non-bitterling

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282   host species was high and ranged from 57.6 to 87.5% (Table 1), implicating that all Asian non-

283   bitterling fish included in the study were physiologically suitable hosts for *S. woodiana*. In contrast,

284   most attached glochidia were shed from both bitterling fish species tested in the first few days post

285   infection, resulting in low metamorphosis success rates of 1.4 - 3.5% (Table 1). Comparing

286   metamorphosis success rate data between native and non-native ranges revealed that an

287   intermediate level of metamorphosis success rate was recorded in Europe for both the bitterling

288   (metamorphosis success 21.7 ± 13.1%) and non-bitterling species (metamorphosis success 43.4 ±

289   15.2%) (mean ± s.d.). The statistical significance of pairwise comparisons is shown in Fig. 2.

290

291   3.2 | *Sinanodonta woodiana* host compatibility at population level

292   The number of initially attached *S. woodiana* per gram of fish body weight (recorded at the beginning

293   of the monitoring period, 30 minutes after infection) differed due to variation among host fish strains

294   ($\chi^2 = 561.3$, *p* < 0.001) but not between *S. woodiana* populations (Table 2; $\chi^2 = 3.78$, *p* = 0.150).

295   Bitterling strains had considerably lower numbers of attached glochidia 30 minutes after infection

296   compared to the control fish species (Fig. 3). There was also a significant interaction between the

297   source mussel population and fish strain ($\chi^2 = 38.3$, *p* < 0.001) and a significant effect of the source

298   mussel individual ($\chi^2 = 15.4$, *p* < 0.001).

299   The infection success of *S. woodiana* demonstrated a similar pattern to the initial attachment rate. It

300   differed due to the variation among host fish strains (GLMM: $\chi^2 = 296.6$, *p* < 0.001) but not between *S.*

301   *woodiana* populations (Table 2; $\chi^2 = 3.36$, *p* = 0.190). The results corroborated the outcome of the

302   species-level experiment; the control fish species *G. rarus* and *R. giurinus* had considerably higher

303   infection rates by *S. woodiana* than all bitterling strains tested (Fig. 3). Although the three *S. woodiana*

304   populations did not differ in their capacity to infect their hosts, there was a significant interaction

305   between source mussel population and fish strain ($\chi^2 = 28.4$, *p* < 0.001). A significant effect of random

306   factors indicated an additional role of individual-level variability among individual fish ($\chi^2 = 463.14$, *p* <

307   0.001) and infections from a specific source of mussels ($\chi^2 = 7.33$, *p* < 0.01). There were no

308   indications of elevated absolute attachment rate or higher infection success in the sympatric mussel-

309   fish combinations (i.e. fish strains and mussel population from Lake Bao'an) (Fig. 3A,B).

310

311   3.3 | Natural prevalence of *S. woodiana* glochidia on host fish

312   In total, 123 fish belonging to 10 species (including 6 bitterling species) were captured and dissected

313   in the native range site (Lake Bao'an) of *S. woodiana* and 243 fish from 10 species (including 1

314   bitterling species) in its non-native range (River Kyjovka). Glochidia were attached to the fins, gills and

315   other body parts (mouths, opercula) of fish. Summary results of glochidia quantification are presented

316   in Table 3. The highest glochidia prevalence per species reached 24% for *R. giurinus* and 78% for *C.*

317   *carpio* at Lake Bao'an and River Kyjovka respectively. The maximum number of *S. woodiana* glochidia

318   per individual fish was 45 larvae recovered from *P. parva* (fish body weight 7.7 g) from the River

319   Kyjovka.

320   In the native range the proportion of bitterling fish parasitized by *S. woodiana* was significantly lower

321   than the proportion of parasitized non-bitterling fish species (Fisher's Exact Test for Count Data: n =

322   123, *p* = 0.003). In contrast, there were no differences between the proportion of parasitized bitterling

323   and non-bitterling fishes in the non-native European range (n = 243, *p* = 0.570).

324

325   **4 | DISCUSSION**

326   4.1 | The role of fish counter adaptations against glochidia

327   This study identifies clear differences in the patterns of bitterling fish utilization by *S. woodiana*

328   glochidia between populations in its native and non-native range. The capacity of *S. woodiana* to

329   exploit non-bitterling fish is relatively high in both areas indicating low dependence on coevolutionary

330   history with specific host taxa. Using the example of European unionids, Bauer (2001) proposed that

331   host specificity of freshwater mussels is primarily determined by available host range. Mussel species

332   that occur in specific habitats with a relatively homogenous fish fauna tend to be host specialists, while

333   species from more diverse and species-rich habitats are host generalists (Bauer, 2001). Thus, a host

334   generalist strategy is supposed to be advantageous in less predictable environments with a diverse

335   and variable fish community, where the survival of glochidia is increased by their ability to utilize a

336   wide range of fish species. At the same time, host generalism may be costly as generalist parasites

337   are often less capable of exploit their hosts efficiently in comparison with specialist parasites (Leggett,

338   Buckling, Long, & Boots, 2013). Earlier studies document that the glochidia of host generalist

339   freshwater mussels are produced in lower numbers than host specialists, though they are larger and at

340   a more advanced stage of development. Notably, they do not grow on their host fish and detach

341   earlier than glochidia of specialists, possibly before the host specific immune response can be initiated

342   (Bauer, 2001). From this perspective, *S. woodiana* expresses life-history traits predicted for a high

343   level of host generalism (Douda, Vrtílek, Slavík, & Reichard, 2012; Dudgeon & Morton, 1984; Hua, Xu,

344   & Wen, 2005) and conclusion supported in the present study based on non-bitterling host species.

345   While Bauer's (2001) concept provides insights into the selective forces driving host range evolution in

346   freshwater mussels, Haag (2012) demonstrated several notable exceptions from the general pattern of

347   generalist-specialist continuum in the diverse North American mussel fauna. Similarly, the present

348   study demonstrated that host generalism in *S. woodiana* has its limitations. Contrasting the two sets of

349   data – experimental exposure (Table 1, Fig. 3) and field-collected data (Table 3) between the native

350   and invasive range (Figure 2, Table 3) revealed that evolutionarily sympatric bitterling species, which

351   face an unusually high risk of parasitism by *S. woodiana*, were inferior hosts for *S. woodiana* glochidia

352   in its native range (China). In contrast, an evolutionarily naïve bitterling species (*R. amarus*) was

353   readily parasitized by *S. woodiana* in its non-native range (Europe). This finding indicates that *S.*

354   *woodiana* can temporarily capitalize on the coevolutionary naivety of hosts during range expansion.

355   Interestingly, significant temporal shifts in host quality have already been recorded after a few

356   generations (Reichard et al., 2015), with European bitterling sympatric with *S. woodiana* for little more

357   than a decade in European waters becoming less suitable hosts for *S. woodiana*.

358   Results from this study do not support the hypothesis that the outcome of the mussel-fish relationship

359   is exclusively determined by mussel adaptations without fish counter-adaptations. The glochidia of *S.*

360   *woodiana* were able to overcome the immune system of almost all hosts tested, including completely

361   allopatric species. However, our data also indicate that if the risk of parasitism is consistently high

362   (such as for the bitterling fishes in *S. woodiana*'s native range), fish are capable of evolving efficient

363   resistance to glochidia. The lack of compatibility with host species that experience high encounter

364   rates with *S. woodiana* glochidia in their natural habitat contradicts predictions from the encounter

365   selection hypothesis.

366 Both innate and acquired host immunity was identified by previous studies to be involved in the

367 protection of fish against glochidia of freshwater mussels (Bauer, 2001; Dodd, Barnhart, Rogers-

368 Lowery, Fobian, & Dimock, 2006; Rogers-Lowery, Dimock, & Kuhn 2007), including *S. woodiana*

369 (Donrovich et al. 2017). In the present study, we were not able to separate the roles of innate and

370 acquired immunity in bitterling fish resistance because we used wild-caught individuals for which we

371 cannot exclude potential previous contact with glochidia. Nevertheless, the high metamorphosis

372 success rate of *S. woodiana* glochidia on control fish species collected from the same sampling site

373 (*R. giurinus*) together with low glochidia prevalence on the host fishes in that collection site (Table 3)

374 indicate a relatively minor role of potential previous contact with glochidia. This assumption is

375 supported by the fact that in a comparable study of host compatibility of European bitterling and *S.*

376 *woodiana*, field collected fish were also used and the study showed high metamorphosis success

377 rates of glochidia (Reichard et al., 2015). Also, natural infection levels of *S. woodiana* on bitterling and

378 non-bitterling fish in both study areas are congruent with the experimental data. However, a different

379 experimental approach will be needed to compare the roles of innate and acquired immunity in

380 bitterling resistance to mussel parasitism.

381 The benefits of fish immunological resistance to glochidia can be relatively small because hosting

382 these short-term parasites can incur a comparable cost to sloughing them (Barnhart, Haag, & Roston

383 2008). Our finding that evolutionarily sympatric bitterling fishes are protected against *S. woodiana*

384 glochidia indicates that such resistance can be cost-effective and that the fitness cost imposed by

385 parasitism is higher than the cost of the response. An alternative explanation is that *S. woodiana*

386 glochidia actively prefer other fish taxa as hosts, with apparent resistance of sympatric bitterling an

387 artefact. However, this explanation is unlikely given the large range of hosts used in our experiment

388 and in previous studies (Dudgeon & Morton, 1984; Hua, Xu, & Wen, 2005; Reichard et al., 2015).

389 Effective resistance by bitterling to glochidia is striking in the context of the potentially mutualistic

390 relationship between bitterling and freshwater mussels. Supporting successful development of

391 glochidia will lead to the establishment of new spawning sites for bitterling. The hypothesis of a

392 mutualistic relationship between the bitterling and mussels has been repeatedly questioned (reviewed

393 in Smith, Reichard, Jurajda, & Przybylski, 2004). Our results corroborate earlier rejection of the

394 hypothesis (e.g. Reichard, Ondračková, Przybylski, Liu, & Smith, 2006; Spence & Smith, 2013). Thus,

395 low prevalence of glochidia on bitterling fish implies a negligible benefit to individual bitterling in acting

396 as mussel hosts. How bitterling avoid glochidial infection, either through behavioural or morphological

397 adaptations, or whether they lose glochidia after a brief initial attachment through an immune response

398 (Smith, Reichard, Jurajda, & Przybylski, 2004), is not known. Whatever the mechanism, based on the

399 present study and the results of Reichard et al. (2015), it is apparent that the coevolutionary

400 relationship between freshwater mussels and bitterling fishes is driven by an antagonistic arms races

401 rather than a mutualistic coevolutionary equilibrium.

402 Overall, our data indicate that fish can evolve a physiological resistance to the glochidia of a highly

403 infective generalist mussel with a short-term parasitic phase. It can be hypothesized that similar

404 resistance can occur in other mussel-fish species combinations. More detailed studies are needed to

405 understand the evolution of host utilization by unionid bivalves, as both mussel adaptation and specific

406 host resistance are likely to be involved in mussel-fish physiological compatibility.

407

408 4.2 | The role of local adaptations in mussel-fish relationships

409 The population scale experiment presented in this study focused on differences in host compatibility

410 between three native range populations of *S. woodiana* inhabiting discrete River Yangtze sub-basins

411 in its native range. The results of this common-garden experiment are in agreement with the previous

412 findings indicating that different mussel populations can vary in their compatibility with particular host

413 strains/populations (Douda et al., 2014; Eckert, 2003; Engel & Wachtler, 1989; Karlsson, Larsen, &

414 Hindar, 2013; Reichard et al., 2015; Rogers, Watson, & Neves, 2001; Serb & Barnhart, 2008). Our

415 finding is unique in documenting population-level variation in host compatibility even for a host

416 generalist taxon within a single river system. This finding adds support for the importance of host-

417 affiliate studies in species management unit recognition (Douda et al., 2014).

418 We hypothesized that the bitterling-glochidia relationship may be population-specific. However, we did

419 not detect significant differences in the ability of *S. woodiana* glochidia to infect sympatric and

420 allopatric populations of *R. ocellatus*, as predicted under the encounter selection hypothesis (Eckert,

421 2003; Rogers, Watson, & Neves, 2001). There was also no support for adaptive differences in host

422 compatibility at the population scale in our study system. These results, together with other recently

423 published evidence (Caldwell, Zanatta, & Woolnough, 2016; Douda et al., 2014; Österling & Larsen,

16

424 2013; Reichard et al., 2015) indicate that specific local adaptations (i.e. between coexisting

425 populations) of mussels and fish are not as common in contemporary communities as previously

426 suggested. However, the lack of evidence for specific local adaptations does not imply that these

427 patterns do not evolve in less dynamic systems with restricted gene flow in mussel and fish

428 populations, or that they have not been common in the past. It is possible that many local adaptations

429 have been eroded through human influence on freshwater biota. *Sinanodonta woodiana* is an

430 economically important species, used as a food source and for other purposes in its native range

431 (Chen, Liu, Su, & Yang, 2015). Transfer of mussels within their native range are common in south-east

432 Asia (Douda, personal observation) and the situation can be even more pronounced for its host fishes.

433 In conclusion, our study contributes to the understanding of the potential role of local adaptations and

434 host resistance in shaping freshwater mussel-fish relationships. The inter-continental spread of *S.*

435 *woodiana*, a host generalist, provides evidence for the role of fish counter-adaptations against

436 glochidia infection, documented in laboratory experiments and directly quantified under natural

437 conditions. The current pace of biotic homogenization of freshwater ecosystems forces many species

438 of freshwater mussel and fish to interact with novel partners (Douda et al., 2013; Poos, Dextrase,

439 Salonen, Marjomäki, & Taskinen, 2016; Schwalb, & Ackerman, 2010; Šlapanský, Jurajda, & Janáč,

440 2016; Tremblay, Morris, & Ackerman, 2016) and local adaptations and counter-adaptations may play a

441 significant role in the quality of host resources available for particular mussel species. Given the global

442 decline of freshwater mussels (Lopes-Lima et al., 2014), including native *S. woodiana* populations in

443 China (Chen et al., 2015), further studies are needed to investigate the impact of host limitation.

444 Unionid bivalves represent functionally important components of freshwater systems and the

445 vulnerability of their host-affiliate relationships will determine the future role of these species in

446 changing freshwater ecosystems. A better understanding of how natural selection drives the evolution

447 of mussel infectivity and fish resistance will be crucial for predicting the impact of biotic

448 homogenization on freshwater mussels. Ultimately, it may lead to better protection of host resources

449 for this endangered taxon.

450

451

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17

458 **RERERENCES**

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625

626 **TABLE 1.** Results of *Sinanodonta woodiana* host compatibility test at the species level. The number of

627 host fish individuals (N_H), mean (± s.d.) values of fish body weight (W), standard length (SL), the

628 number of glochidia attached on individual fish(N_G), and metamorphosis success (M) are shown.

629 Attached glochidia represent the sum of glochidia and metamorphosed juveniles recovered from

630 individual fish; metamorphosis success indicates % of attached glochidia that were recovered as live

631 juveniles. Mean values denoted by the same superscript letter are not significantly different (Tukey's

632 HSD test at $p < 0.05$). Bitterling hosts highlighted in bold.

Species	N_H	W (g)	SL (mm)	N_G	M (%)
***Rhodeus ocellatus* (Kner, 1866)**	**26**	**0.83 ± 0.36**	**34.3 ± 3.9**	**57.7 ± 59.0**	**1.4 ± 3.7** [C]
***Rhodeus sinensis* Günther, 1868**	**11**	**1.18 ± 0.48**	**37.7 ± 4.5**	**87.8 ± 61.1**	**3.5 ± 4.2** [C]
Carassius auratus (Linnaeus, 1758)	8	0.37 ± 0.20	24.7 ± 4.4	55.5 ± 15.0	85.4 ± 3.8 [A]
Danio rerio (Hamilton, 1822)	10	0.37 ± 0.04	27.8 ± 0.5	63.2 ± 18.7	65.0 ± 15.4 [AB]
Rhinogobius giurinus (Rutter, 1897)	11	0.94 ± 0.20	41.2 ± 2.5	175.1 ± 73.6	60.0 ± 21.2 [B]
Hypophthalmichthys nobilis (Richardson, 1845)	6	0.20 ± 0.06	21.7 ± 3.6	21.8 ± 13.4	87.5 ± 6.9 [AB]
Sinibotia superciliaris (Günther, 1892)	10	1.01 ± 0.24	41.6 ± 3.2	96.3 ± 22.5	57.6 ± 16.0 [B]
Silurus meridionalis Chen, 1977	7	1.42 ± 0.88	55.4 ± 12.1	272.0 ± 114.5	57.9 ± 9.9 [B]
Plagiognathops microlepis (Bleeker, 1871)	10	0.13 ± 0.03	20.7 ± 2.0	18.8 ± 5.7	75.2 ± 13.6 [AB]

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635 **TABLE 2.** Sources of variation in host compatibility between *Sinanodonta woodiana* and its native-

636 range hosts. Effects of multiple variables on the number of attached glochidia per gram of fish body

637 weight (using *lmer* in R) and infection success (using *glmer* in R). The results show the effects of fixed

638 factors *Mussel population* (populations A - Jianli, B - Bao'an, C - Qinglan), *Fish strain* (sympatric *R.*

639 *ocellatus,* allopatric *R. ocellatus, R. sinensis, R. giurinus, G. rarus*) and their interaction, as well as

640 random effects of *Mussel individual* and *Host fish individual*. Chi-square values, degrees of freedom

641 (DF), and *P*-values are shown for the likelihood-ratio tests between full models and models with each

642 factor removed. The effects that improved the model fit significantly ($p < 0.05$) are highlighted in bold.

	A. Attached per gram		B. Infection success	
	X^2 (DF)	*p*-value	X^2 (DF)	*p*-value
Fixed Factors				
Mussel pop.	3.78 (2)	0.15	3.36 (2)	0.19
Fish strain	561.3 (4)	**<0.001**	296.6 (4)	**<0.001**
Mussel pop. x Fish strain	38.3 (8)	**<0.001**	28.4 (8)	**<0.001**
Random Factors				
Mussel ind.	15.4 (1)	**<0.001**	7.3 (1)	**<0.01**
Host fish ind.	-	-	463.1 (1)	**<0.001**

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649 **TABLE 3** The natural prevalence and intensity of infection of *Sinanodonta woodiana* glochidia on fish

650 collected from the field during the glochidia discharge season at study populations from its native

651 (Lake Bao'an; China) and non-native range (River Kyjovka; Czech Republic). The number of host

652 individuals (N_H), mean (± s.d.) values of fish body weight (W), standard length (SL), the number of

653 infected hosts (N_{INF}), total number of glochidia on all fish (N_{GT}), mean intensity of infection (Intens) and

654 glochidia prevalence (Prev) are shown. Prevalence indicates the proportion of fish infected. Mean

655 intensity of infection indicates the average number of *S. woodiana* glochidia per infected host.

656 Bitterling hosts highlighted in bold.

	Fish species	N_H	W (g)	SL (mm)	N_{INF}	N_{GT}	Intens	Prev
Native range	***Acheilognathus barbatulus* Günther, 1873**	35	2.17 ± 0.86	45.7 ± 6.2	2	2	1.00	0.06
	***Acheilognathus macropterus* (Bleeker, 1871)**	1	3.55	57	0	0	-	0.00
	***Acheilognathus tonkinensis* (Vaillant, 1892)**	3	2.62 ± 0.59	49.0 ± 5.0	0	0	-	0.00
	***Paracheilognathus imberbis* (Günther, 1868)**	6	1.22 ± 0.46	39.8 ± 5.6	0	0	-	0.00
	***Rhodeus sinensis* Günther, 1868**	2	0.87, 0.68	32, 29	0	0	-	0.00
	***Rhodeus ocellatus* (Kner, 1866)**	33	0.99 ± 0.33	33.2 ± 4.2	0	0	-	0.00
	Carassius auratus (Linnaeus, 1758)	12	0.80 ± 0.32	31.4 ± 4.5	2	3	1.50	0.17
	Hemiculter leucisculus (Basilewsky, 1855)	2	2.54, 2.92	69, 73	0	0	-	0.00
	Mastacembelus aculeatus (Bloch, 1786)	8	4.56 ± 3.08	120.1 ± 13.2	1	1	1.00	0.13
	Rhinogobius giurinus (Rutter, 1897)	21	0.82 ± 0.19	34.9 ± 3.1	5	6	1.20	0.24
Introduced range	***Rhodeus amarus***	96	0.45 ± 0.79	19.6 ± 10.1	26	45	1.73	0.27
	Blicca bjoerkna (Linnaeus, 1758)	23	7.45 ± 3.90	64.2 ± 11.3	3	3	1.00	0.13
	Alburnus alburnus (Linnaeus, 1758)	25	4.75 ± 2.58	65.9 ± 10.3	0	0	-	0.00
	Scardinius erythrophthalmus (Linnaeus, 1758)	23	6.52 ± 3.38	62.8 ± 11.0	11	48	4.36	0.48
	Rutilus rutilus (Linnaeus, 1758)	11	6.88 ± 3.62	65.6 ± 16.4	0	0	-	0.00
	Pseudorasbora parva (Temminck &	31	2.70 ± 2.26	46.5 ± 14.8	14	127	9.07	0.45

Schlegel, 1846)

Perca fluviatilis Linnaeus, 1758	2	8.19, 4.62	68, 59	1	3	3.00	0.50
Gobio gobio (Linnaeus, 1758)	11	3.42 ± 2.19	55.9 ± 14.0	2	4	2.00	0.18
Cyprinus carpio Linnaeus, 1758	9	6.13 ± 1.25	52.9 ± 3.9	7	33	4.71	0.78
Carassius gibelio (Bloch, 1782)	12	4.57 ± 4.08	44.5 ± 13.8	8	34	4.25	0.67

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658   **FIGURE CAPTIONS**

659   **FIGURE 1** Study area with collection sites in the *Sinanodonta woodiana* native (A – Jianli, B - Lake

660   Bao'an, C - Lake Qinglan; China) and non-native range (D – River Kyjovka; Czech Republic).

661   **FIGURE 2** Metamorphosis success rate of *Sinanodonta woodiana* (population B - Lake Bao'an; China)

662   on tested East Asian host fish species (median (dashed line), interquartile range (box), min/max

663   (whiskers), and outliers > 1.5 x interquartile range displayed). Data from a previously tested non-native

664   population (Douda, Vrtílek, Slavík, & Reichard, 2012) are also included for a comparison (grey boxes).

665   Bitterling fishes (*R. sinensis*, *R. ocellatus* and *R. amarus*) are represented by hatched boxes. Boxes

666   with different index letters (A, B, C) are statistically significantly different from each other (Tukey's HSD

667   test, $p < 0.05$).

668   **FIGURE 3** Population-level host compatibility of *Sinanodonta woodiana* with its native range hosts.

669   The number of attached glochidia per gram of fish body weight (A) and infection success (B) versus

670   *Mussel population* (populations A - Jianli, B - Bao'an, C - Qinglan) and *Fish strain* in the population-

671   level experiment. Results of GLMM with the respective *p*-values for the effects of *Mussel population*,

672   *Fish strain* and the interaction term are displayed (n = 14-16 per group).

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