

How to cite this article: Douda K, Liu H-Z, Yu D, et al. The role of local adaptation in shaping fish-mussel coevolution. *Freshwater Biol.* 2017;00:1–11. <https://doi.org/10.1111/fwb.13026>

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

2 Running title: Freshwater mussel-host coadaptation

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24 **KEYWORDS**

25 affiliate species, glochidia, host relationships, freshwater bivalves, *Sinanodonta woodiana*

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 (Campiã, Ribas,
63 Cornell, Begon, & Tavaréz, 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

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.

141

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 \pm 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 \pm s.d.) during the experiment. Water in
176 individual tanks was filtered through nylon screens (mesh size 139 μ m; 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 \pm s.d. standard
209 length and mean \pm 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 \pm 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 \pm 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

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 $21.7 \pm 13.1\%$) and non-bitterling species (metamorphosis success $43.4 \pm$
289 15.2%) (mean \pm 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,

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

452 **ACKNOWLEDGEMENTS**

453 The authors thank Radim Blažek, Věra Plechingerová, Milan Vrtílek, Matej Polačik, Klára Matasová
454 and Eva Karbanová for help with data collection and experimental procedures and two anonymous
455 referees for constructive comments. All experimental procedures were approved by the Expert
456 Committee for Animal Conservation (research protocol no. MSMT-31220/2014-6). The research was
457 funded by the Czech Science Foundation (13-05872S).

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

633

634

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	
	χ^2 (DF)	<i>p</i> -value	χ^2 (DF)	<i>p</i> -value
Fixed Factors				
<i>Mussel pop.</i>	3.78 (2)	0.15	3.36 (2)	0.19
<i>Fish strain</i>	561.3 (4)	<0.001	296.6 (4)	<0.001
<i>Mussel pop. x Fish strain</i>	38.3 (8)	<0.001	28.4 (8)	<0.001
Random Factors				
<i>Mussel ind.</i>	15.4 (1)	<0.001	7.3 (1)	<0.01
<i>Host fish ind.</i>	-	-	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 (\pm 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	<i>Acheilognathus barbatulus</i> Günther r, 1873	35	2.17 \pm 0.86	45.7 \pm 6.2	2	2	1.00	0.06
	<i>Acheilognathus macropterus</i> (Bleeker, 1871)	1	3.55	57	0	0	-	0.00
	<i>Acheilognathus tonkinensis</i> (Vaillant, 1892)	3	2.62 \pm 0.59	49.0 \pm 5.0	0	0	-	0.00
	<i>Paracheilognathus imberbis</i> (Günther, 1868)	6	1.22 \pm 0.46	39.8 \pm 5.6	0	0	-	0.00
	<i>Rhodeus sinensis</i> Günther, 1868	2	0.87, 0.68	32, 29	0	0	-	0.00
	<i>Rhodeus ocellatus</i> (Kner, 1866)	33	0.99 \pm 0.33	33.2 \pm 4.2	0	0	-	0.00
	<i>Carassius auratus</i> (Linnaeus, 1758)	12	0.80 \pm 0.32	31.4 \pm 4.5	2	3	1.50	0.17
	<i>Hemiculter leucisculus</i> (Basilewsky, 1855)	2	2.54, 2.92	69, 73	0	0	-	0.00
	<i>Mastacembelus aculeatus</i> (Bloch, 1786)	8	4.56 \pm 3.08	120.1 \pm 13.2	1	1	1.00	0.13
	<i>Rhinogobius giurinus</i> (Rutter, 1897)	21	0.82 \pm 0.19	34.9 \pm 3.1	5	6	1.20	0.24
Introduced range	<i>Rhodeus amarus</i>	96	0.45 \pm 0.79	19.6 \pm 10.1	26	45	1.73	0.27
	<i>Blicca bjoerkna</i> (Linnaeus, 1758)	23	7.45 \pm 3.90	64.2 \pm 11.3	3	3	1.00	0.13
	<i>Alburnus alburnus</i> (Linnaeus, 1758)	25	4.75 \pm 2.58	65.9 \pm 10.3	0	0	-	0.00
	<i>Scardinius erythrophthalmus</i> (Linnaeus, 1758)	23	6.52 \pm 3.38	62.8 \pm 11.0	11	48	4.36	0.48
	<i>Rutilus rutilus</i> (Linnaeus, 1758)	11	6.88 \pm 3.62	65.6 \pm 16.4	0	0	-	0.00
	<i>Pseudorasbora parva</i> (Temminck &	31	2.70 \pm 2.26	46.5 \pm 14.8	14	127	9.07	0.45

Schlegel, 1846)

<i>Perca fluviatilis</i> Linnaeus, 1758	2	8.19, 4.62	68, 59	1	3	3.00	0.50
<i>Gobio gobio</i> (Linnaeus, 1758)	11	3.42 ± 2.19	55.9 ± 14.0	2	4	2.00	0.18
<i>Cyprinus carpio</i> Linnaeus, 1758	9	6.13 ± 1.25	52.9 ± 3.9	7	33	4.71	0.78
<i>Carassius gibelio</i> (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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