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

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

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Introduction

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

Most host–parasite relationships convey significant costs to both partners and result in a rapid coevolutionary arms race when an adaptation in one partner is followed by reciprocal counter-adaptations in the other (Dawkins and Krebs 1979). This process is dynamic and at a particular evolutionary point, hosts may be better adapted to defend themselves from the parasite or, alternatively, the parasite can be better adapted to exploit its host (Rothstein and Robinson 1998; Sorenson et al. 2003; Medina and Langmore 2016). *The parasite specialization scenario* considers that the present coevolutionary state is primarily resolved by traits evolved by the parasite, with parasite adaptations currently overcoming the defences evolved by the host. The *host resistance scenario* predicts that the present coevolutionary state is primarily resolved by the host, which expresses adaptations that resist parasite virulence (Davies and Brooke 1988; Honza et al. 2004). An alternative possibility is that adaptive responses in the host–parasite relationship are lacking, a situation predicted when the cost, probability or intensity of an interaction is limited (Holland et al. 2004).

Parasites may specialise on particular host species (Joshi and Thompson 1995) or specific host populations (Kaltz and Shykoff 1998; Kawecki and Ebert 2004). Host communities vary in their species composition, relative abundance of each host species and density of their populations. Given that each host species may require different parasite adaptations, variation in the structure of local host communities may lead to *parasite specialization at the local level*, often to the locally most common host species (Thompson 1994, 1999; Krasnov et al. 2011). This situation produces variable host–parasite associations across geographic regions, leading to local differences in parasite specialization. An example is the European cuckoo (*Cuculus canorus* L.) where different populations specialize on different locally accessible host species (Medina and Langmore 2016).

In other host–parasite systems, however, parasites may coevolve with a single host species across their entire range. *Parasite specialization at the species level* requires generalised adaptations to exploit a host species and may be favoured when either parasite or host species disperse over large distances (Medina and Langmore 2016). In African indigobirds (*Vidua* spp.), strict host–parasite association at the species level is driven by precise mouth marking in chicks that mimic mouth marking of their respective host species (Sorenson et al. 2003). Stronger specialization on one host species, therefore, comes at a cost of a decrease in fitness on other host species, leading to parasite specialisation at the level of the host species. High host specificity is expected to lead to more intense escalation of reciprocal adaptations in hosts and parasites.

An analogous, though reversed, scenario is the situation when host resistance dominates the current state of the reciprocal relationship. Increased resistance to parasites can improve a host's fitness, but is often costly as resources are diverted from growth and reproduction (Moret and Schmid-Hempel 2000; Rigby and Moret 2000; Schmid-Hempel 2003). Hence, a high investment in host resistance is more likely to be maintained under intense parasite pressure. When effective host responses are detected, *host resistance* can be defined at the species and local population levels, similarly to parasite specialization.

A powerful way to test for host and parasite adaptation is by using cross-infection experiments to compare parasite success (or preference) and host resistance for sympatric versus allopatric populations (reviewed by Kawecki and Ebert 2004). *Local parasite adaptation* can be detected by measuring the fitness of a parasite on locally coexisting host populations, with a prediction of parasite preference and increased fitness on locally coexisting hosts. In contrast, *local host resistance* predicts increased parasite fitness on ecologically naive hosts and, in the case that the parasite is capable of evaluating host suitability, parasite preference for ecologically naive host populations over coexisting host populations (Kawecki and Ebert 2004). Previous studies have provided evidence for local adaptation of the parasite, local adaptation of the host, and no local adaptation (Edmunds and Alstad 1978; Greischar and Koskella 2007; Hoeksema and Forde 2008; Laine 2009; Voutilainen et al. 2009).

In this study, we investigated interpopulation variation in host–parasite association between a parasitic fish, the rose bitterling, *Rhodeus ocellatus* (Kner), and their host mussels from the family Unionidae. *Rhodeus ocellatus*, like all other bitterling fishes (family Acheilognathidae), lay their eggs in the gills of unionid mussels. Female bitterling use a long ovipositor to lay their eggs in the gills of living mussels by inserting them into the host's exhalant siphon. Male bitterling fertilize the eggs deposited inside the mussel gill by releasing sperm over the host inhalant siphon (Reichard et al. 2004a; Smith and Reichard 2013). Males defend a territory containing one or more mussels against rivals, actively court females and lead them to a mussel (Smith et al. 2004). Bitterling embryos develop in the gills of the host mussel, competing for nutrients and oxygen with the host, before emerging after 3–4 weeks (Aldridge 1999; Spence and Smith 2013). Hosting bitterling embryos is costly to mussels (Reichard et al. 2006), and mussels have evolved counter-adaptations to bitterling parasitism, primarily ejections of their eggs and embryos (Kitamura 2005; Reichard et al. 2007a, b, 2010). In turn, bitterling eggs and embryos have evolved behavioural and morphological mechanisms to avoid ejection (Smith et al. 2004). The bitterling-mussel system is conceptually analogous to avian brood parasitism (Davies 2015).

Rhodeus ocellatus is widely distributed across East Asia (Chang et al. 2014). It is a generalist parasite utilising a range of host mussel species, but exhibiting preferences for particular hosts (Reichard et al. 2007a, b). Female *R. ocellatus* display preferences toward certain mussel individuals (Casalini et al. 2013), indicating that female *R. ocellatus* may tailor their host preferences to host quality cues. At least at the level of individual host mussels, males appear less selective than females (Casalini et al. 2013), trading off mussel quality against the risk of sperm competition (Smith et al. 2003). Although female oviposition decisions are also affected by mate quality (Agbali et al. 2010, 2012), the quality of the host mussel is the key factor predicting female oviposition-site decisions (Casalini et al. 2009). Female host choice decisions in *R. ocellatus* and other bitterling species rely on optimal mussel traits related to enhanced embryo survival, particularly the availability of oxygen for developing embryos (Smith et al. 2001; Reichard et al. 2007a, b; Spence and Smith 2013; Phillips et al. 2017).

There is an indication for coevolutionary dynamics in the relationship between bitterling fishes and their mussel hosts. Populations of the related European bitterling, *Rhodeus amarus* (Bloch), display variation in host species preference across their geographic distribution (Reichard et al. 2010; Smith 2017). Similarly, host mussels have evolved stronger defences (e.g. egg ejection) in areas of ancient sympatry with *R. amarus* than in areas of recent sympatry (Reichard et al. 2010). Hence, bitterling and their hosts can exhibit population-level variation in the intensity of reciprocal selection. We hypothesized that this variation may lead to different degrees of local adaptation, especially in areas of ancient sympatry, driven either by parasite adaptation or host resistance.

Here, we used two geographically distant populations of *R. ocellatus* from a region of ancient bitterling-mussel sympatry to test their preference towards two widespread host mussel species, *Anodonta woodiana* (Lea) and *Unio douglasiae* (Gray). Despite wide co-occurrence between both mussel host species and *R. ocellatus*, *A. woodiana* hosts have a larger distribution and are locally more abundant, typically being the dominant unionid species in mussel communities throughout the range of *R. ocellatus* (He and Zimin 2013). Each host mussel species was represented by one population coexisting with and one population naïve to *R. ocellatus*. Mussel host populations varied in their level of regional and local coexistence with *R. ocellatus*, producing a mosaic of associations at the species and local levels (Fig. 1). We also measured population-specific host mussel resistance to *R. ocellatus* oviposition through egg ejections.

The coevolutionary relationship between bitterling and their mussel hosts is antagonistic and dynamic (Smith et al. 2004; Reichard et al. 2012; Spence and Smith 2013). For *parasite specialisation* to play a dominant role in the association the prediction is that *R. ocellatus* will make oviposition-site decisions that favour the utilization of mussels with which there is an evolutionary association at a meta (species) level, as well as at a local (population) level. In contrast, in the case that *host resistance* plays the primary role
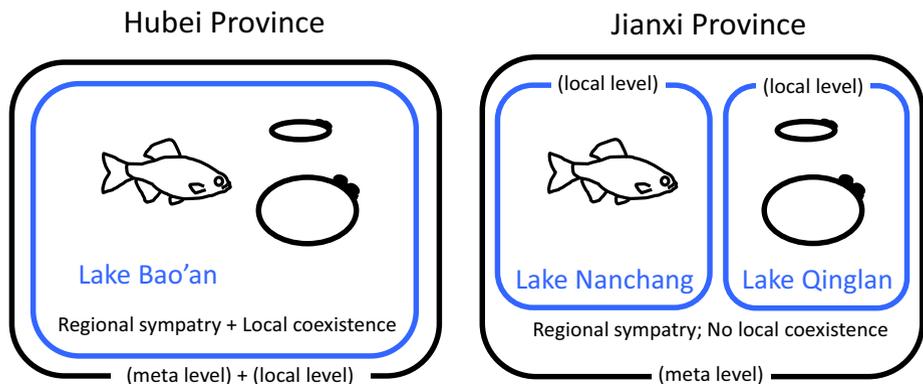
Fig. 1 An illustration of experimental populations with regard to their reciprocal coexistence at a meta-level (sympatry/allopatry) and local coexistence (locally coexisting/ecologically naïve). Each experimental pair of *Rhodeus ocellatus* was tested against four contrasting pairs of host mussels, with all four trials completed over 1–3 days. The trials represented contrasts between (1) *Anodonta woodiana* from two populations; and (2) *Unio douglasiae* from two populations, each contrasting geographically sympatric and allopatric hosts; (3) *A. woodiana* and *U. douglasiae* from Lake Bao'an, contrasting host species that both locally coexisted with *R. ocellatus*; and (4) *A. woodiana* and *U. douglasiae* from Lake Qinglan, contrasting host species that were both ecologically naïve to *R. ocellatus*. Note that since two *R. ocellatus* populations were used overall, each contrast had additional predictions that are articulated in Table 1

Table 1 Experimental design showing the predicted responses of parasitic *R. ocellatus* and host mussels for scenarios when parasite specialisation (tested using oviposition preference) or host resistance (tested using egg ejection) dominate the current coevolutionary state

Response variable	Covariate	Covariate level	Predictions		Support
			Parasite specialisation	Host resistance	
Parasite oviposition	Sympatry	Sympatric	Yes	No	Yes
		Allopatric	No	Yes	
Parasite oviposition	Local coexistence	Coexisting	Yes	No	–
		Not coexisting	No	Yes	
Host egg ejection	Sympatry	Sympatric	No	Yes	Yes
		Allopatric	Yes	No	
Host egg ejection	Local coexistence	Coexisting	No	Yes	–
		Not coexisting	Yes	No	

Yes—preference for this host supports the prediction

No—preference for this host contradicts the prediction

in oviposition-site decisions, *R. ocellatus* are predicted to use allopatric mussel species at a meta level and populations without previous exposure to bitterling at a local level. In the context of the present study, in the case of *parasite specialisation at the species level* we predicted a preference by experimental *R. ocellatus* for sympatric host species at a broad (meta) geographic scale and, under the *parasite specialisation at the local level*, a preference for populations with which they locally coexist. In the case of *host resistance at the species level* we predicted a preference for allopatric and locally naive hosts. These predictions assumed that *R. ocellatus* are able to assess relevant cues associated with a host mussel's ability to eject eggs as the European bitterling *R. amarus* do (Mills and Reynolds 2002), though we did not test this assumption. Predictions for each host mussel and bitterling population under each coevolutionary scenario are summarized in Table 1.

Materials and methods

Study subjects

Two species of host mussel (*Anodonta woodiana* and *Unio douglasiae*) were collected from each mussel collection site. These were Lake Bao'an in Hubei Province (30°17′25.4″N; 114°43′48.9″E) and Lake Qinglan (28°30′40.144″N; 116°8′ 2.112″E), Jiangxi Province, China. Both mussel species were confirmed to be hosts of *R. ocellatus* (Reichard et al. 2007a, b; R. Rouchet unpublished data). Mussels locally coexisting with the local *R. ocellatus* population were collected from Lake Bao'an, a shallow lake with abundant aquatic vegetation where *R. ocellatus* is common and co-occurs with at least four other bitterling species—*Rhodeus sinensis* Guenther, *Rhodeus fangi* (Miao), *Acheilognathus chankaensis* (Dybowski) and *Acheilognathus imberbis* Guenther. Mussels naive to *R. ocellatus* were collected from Lake Qinglan, a part of Lake Poyang, the largest lake in China. Bitterling species recorded in Lake Qinglan include *A. chankaensis*, *A. imberbis*,

Acheilognathus tonkinensis (Vaillant), *Acheilognathus* cf. *meridianus* (Wu), *Acheilognathus barbatulus* Guenther, *Acheilognathus rhombeus* (Temminck and Schlegel) and *Acheilognathus gracilis* Nichols. There are no records of *R. ocellatus* from the lake although this species is found in some smaller affiliated waters. Given the pronounced dispersal capability of unionid mussels during their larval stage (Douda et al. 2012), it is possible that host mussels from Lake Qinglan may have a history of coexistence with *R. ocellatus* at a larger scale, despite individual host mussels from Lake Qinglan not co-occurring with *R. ocellatus*. In Lake Qinglan, *U. douglasiae* is known to be parasitized by the embryos of *A. tonkinensis* (a bitterling species unrelated to *R. ocellatus*), while there are no records of bitterling embryos recovered from *A. woodiana* from Lake Qinglan (Liu et al. 2006).

Mussels were collected by hand from both lakes. In the case of Lake Qinglan, because of its greater depth, specimens were also collected using a mussel dredge hauled by a boat. Stocks of experimental *A. woodiana* and *U. douglasiae* were collected in early April 2014 and supplemented during three additional collections in Lake Bao'an and four in Lake Qinglan between late April and mid-June. Mussels were stored in large (2.0 × 1.0 m) plastic containers filled with water to a depth of approximately 300 mm and aerated continuously.

Rhodeus ocellatus used in the study were collected in April and May 2014 using baited fish traps. Fish from the locally coexisting population were collected from Lake Bao'an (the same site as mussel collection) where *R. ocellatus* lived in sympatry with at least four species of freshwater mussel, including both study host species (included as two host study populations). The second population of *R. ocellatus* (Nanchang) was not coexisting with any experimental populations of host mussels, but coexisted with a local (non-experimental) population of *A. woodiana*. Fish from this *R. ocellatus* population were collected in a natural pond in the city of Nanchang (Jiangxi province, 28°39′55.08″N; 115°48′57.79″E), located 35 km from Lake Qinglan where the mussels ecologically naïve to *R. ocellatus* were collected. The pond was shallow (<1 m) and measured approximately 15 × 40 m. There was no connection allowing fish migration between the pond and Lake Qinglan or adjacent bodies of water. Only a single mussel species (*A. woodiana*) was recorded in Nanchang pond despite an intensive search. *R. ocellatus* coexisted in the pond with another bitterling species, *R. sinensis*.

Experimental bitterling were transported in aerated containers and housed in four large (2.0 × 1.0 m) tanks. A maximum of 150 fish were stored in each tank, with approximately equal sex ratio. In each tank we placed *Lamprotula caveata* (Heude) mussels to encourage female ovulation. These mussels were enclosed with transparent covers that permitted bitterling to see and smell them but prevented oviposition. *L. caveata* was chosen because it is phylogenetically and morphologically unrelated to *A. woodiana* and *U. douglasiae* (Liu et al. 2006) and hence prevented fish habituation towards one of the tested host mussels prior to the start of the experiment.

Behavioural experiment

The experiment consisted of a test of male and female *R. ocellatus* towards a pair of host mussel species of different combinations of host species, meta and local co-occurrence (Fig. 1). We use the terms *sympatric* and *allopatric* at the "meta" scale (Bao'an host mussels sympatric with *R. ocellatus*, Qinglan host mussels allopatric with *R. ocellatus*) and the terms *locally coexisting* and *ecologically naïve* at the "local" scale (*ecologically naïve*: populations of fish and mussels not locally coexisting regardless of sympatry at the meta

scale) (Fig. 1). The experiment took place between April and June 2014 in the laboratory facilities of the Institute of Hydrobiology of the Chinese Academy of Sciences in Wuhan, China.

For each replicate, we used a set of four mussels: one *A. woodiana* from Lake Bao'an, one *A. woodiana* from Lake Qinglan, one *U. douglasiae* from Lake Bao'an and one *U. douglasiae* from Lake Qinglan (Fig. 1). Although it was impossible to obtain all four adult mussels of the same size because *A. woodiana* (shell length: 91–132 mm) is larger than *U. douglasiae* (37–91 mm), mussels from the same species within a set were size-matched. Four separate preference tests (always containing a pair of host mussels; Fig. 1) were performed in a random predetermined order. A full set of preference tests within a replicate was tested within one day for one of the two *R. ocellatus* populations (Bao'an or Nanchang). The same set of mussels was tested with the second fish population on a subsequent day, usually the following day, but always within one week. A set of four host mussels (one individual from each study population) was, therefore, tested in four separate paired tests (*A. woodiana* Bao'an × *A. woodiana* Qinglan; *U. douglasiae* Bao'an × *U. douglasiae* Qinglan; *A. woodiana* Bao'an × *U. douglasiae* Bao'an; *A. woodiana* Qinglan × *U. douglasiae* Qinglan) with the same pair of *R. ocellatus* fish in one day and with another pair of *R. ocellatus* (from a different population) on a later day (Fig. 1). In seven cases, mussel mortality prevented completion of the test with the second bitterling population.

Experimental aquaria measured 550 (length) × 350 (width) × 450 (depth) mm and contained a 20 mm layer of washed sand as a substrate. In the evening prior to the start of a test, a focal male *R. ocellatus* was introduced into an experimental aquarium with a non-experimental *L. caveata* mussel placed in the centre of the aquarium to elicit territoriality. On the following morning the *L. caveata* mussel was removed. A second, non-experimental *R. ocellatus* male (from the same population as the focal male) was placed in the tank in a cylindrical glass jar (diameter 100 mm) to further stimulate the territorial and reproductive behaviour of the focal male. A female in spawning condition, obvious from an extended ovipositor that unambiguously demonstrates that a female bitterling has ovulated a batch of eggs, from the same population as the focal male was gently released into the aquarium. The first pair of mussels to be tested were placed 150 mm apart in the centre of the aquarium inside sand-filled plastic pots to hold them in position. The mussels were covered with a transparent pierced plastic box that allowed the fish to see and smell the mussels but prevented oviposition. After at least 30 min of acclimation, the covers were removed from the mussels. Behaviour recording started once one of the experimental fish began inspection of the siphons of a mussel. Behaviours directed at a specific host mussel and relevant to oviposition preference were recorded. These were: male *leading* the female *R. ocellatus* towards one of the mussels (though with no interference with that mussel), male or female *inspection* of the exhalant and inhalant siphons of the host mussel (sampling host cues to assess its suitability for the incubation of *R. ocellatus* embryos), *sperm release* by the *R. ocellatus* male (indicating investment into a particular host mussel, clearly detected by typical male movement over the mussel inhalant siphon), *skimming* by a *R. ocellatus* female (a behaviour resembling oviposition but without insertion of the ovipositor into the mussel siphon and without egg laying) and female *oviposition* (spawning, identified by a typical female movement and insertion of her ovipositor into the mussel exhalant siphon). For a detailed description of bitterling reproductive behaviour see Reichard et al. (2004b). *R. ocellatus* behaviour was recorded continuously for 20 min or until oviposition, whichever occurred earlier. If oviposition occurred, the mussel was observed for a further 1 min to record any egg ejections by the host mussel. Mussel ejections occur either within a few seconds of oviposition (immediate ejections) or over an

extended period of several days (late ejections). The two types of ejections are positively correlated (Reichard et al. 2007a, b) but late ejections cannot be observed directly. After completion of a trial the experimental host mussels were replaced with the next treatment combination and covered until the subsequent behavioural test, with a minimum interval of 30 min between tests to ensure that females were capable of spawning another batch of ovulated eggs (Smith et al. 2004). Experimental mussels did not contain any bitterling eggs or embryos prior to their use in a trial. Once all trials were completed for a given pair of fish, they were stocked in a separate tank and not used again in the experiment.

Data analysis

Data from each focal female and male *R. ocellatus* were used to model host mussel preference. Before applying statistical models a data exploration was undertaken, following the protocol described in Ieno and Zuur (2015). The data were examined for outliers in the response and explanatory variables, homogeneity and zero inflation in the response variable, collinearity between explanatory variables and the nature of relationships between the response and explanatory variables. Oviposition was used as a definitive measure of mussel preference. We detected collinearity between female inspection of host mussels with oviposition and dropped female inspection from the analysis. Male mussel inspection behaviour was similarly found to be collinear with male leading behaviour and was subsequently dropped from the analysis. Male leading behaviour was included in the model as a covariate to accommodate the effect of male behaviour on female oviposition-site decision. Sperm release by males rarely occurred prior to oviposition, limiting its informative value, and this variable was not included in the model. No outliers were detected.

Mussel preference was modelled using a Bernoulli Generalized Linear Mixed Model (GLMM). The model contained fixed effects for *sympatry* (two levels: sympatry and allopatry), *local coexistence* (two levels: locally coexisting and ecological naive), and *host species* (two levels: *A. woodiana* and *U. douglasiae*). An interaction between host mussel species and local coexistence was included to test for an effect of conditioning on host preference. *Male leading* was a covariate representing the frequency of male leading behaviour to a mussel, which can potentially influence female oviposition decisions (Smith et al. 2004). We also included the effects of body size (measured as the total length, including caudal fin) of male and female *R. ocellatus*. Mussel shell size was not included as a covariate because host mussels were size-matched within host species and fully collinear (non-overlapping size range) between species. Random terms were *Fish ID* (permitting random variation on the intercept for each individual fish (fish pair used in trials) and *Trial* as a random intercept for each pairwise experimental trial (with response to two host mussel individuals tested simultaneously in a paired design) nested within individual fish and permitting a different intercept for each experimental trial.

The occurrence of egg ejection by mussels immediately following oviposition was modelled using a subset of the data that included only trials that resulted in oviposition. The model was fitted as a Bernoulli GLMM (log-link function) and contained the same set of fixed and random effects as the oviposition model, except for the removal of *male leading* given the lack of an association between male leading behaviour and host mussel ejection. Data analyses were conducted using the *lme4* package in R 3.2.0 (Bates et al. 2014; R Core Development Team 2014). We quantified model goodness-of-fit (R^2_{GLMM}) for the fixed part of the model (R^2_{GLMM} marginal) and for the complete model (including random effects; R^2_{GLMM} conditional) (Nakagawa and Schielzeth 2013). Goodness-of-fit

parameters were extracted using the *MuMIn* package (Bartoń 2014). The *effects* package (Fox et al. 2016) was used to visualize model estimates of fixed term effects.

Results

A total of 272 paired trials were conducted, of which 80 resulted in oviposition. *Rhodeus ocellatus* spawned more frequently in allopatric than sympatric mussel species, regardless of mussel population of origin (Table 2a; Fig. 2), which is in agreement with a predominant role for *host resistance*. There was no effect of local coexistence between *R. ocellatus* and host mussels on the probability of *R. ocellatus* oviposition, suggesting the parasite specialization was not affected by local interactions. There was no effect of host mussel species on the probability of *R. ocellatus* oviposition, indicating that there was no parasite specialization for a particular host species. There was no interaction between host mussel species and the effect of local coexistence, indicating no effect of local conditioning on host preference. Male leading behaviour, a covariate in the model, had a significant positive effect on the probability of oviposition (Table 2a). There was no effect of female *R. ocellatus* body size on oviposition but a positive trend of male *R. ocellatus* body size (Table 2a), indicating that females were more likely to spawn with larger males. The model explained 9% of variability in the data (R^2_{GLMM} marginal = 0.0887), with almost all explained variation related to fixed part of the model (R^2_{GLMM} conditional = 0.0887).

Ejection of *R. ocellatus* eggs by host mussels showed a significant effect of mussel species (Table 2b), with *A. woodiana* exhibiting a higher frequency of egg ejection than *U. douglasiae* (Fig. 3). Sympatric mussel hosts, irrespective of species, tended to eject *R. ocellatus* eggs more frequently, though this effect was not statistically significant

Table 2 Estimates and *P* values of fixed effects from the GLMMs on the oviposition rate (a) and counts of egg ejection (b)

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

Significant *P* values are indicated in bold
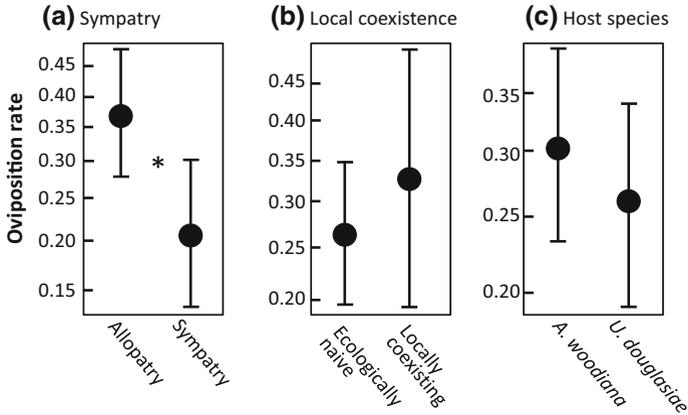
Fig. 2 Mean rate of oviposition of female *R. ocellatus* with respect to **a** sympatry/allopatry between *R. ocellatus* (parasite) and host mussel populations, **b** local coexistence between *R. ocellatus* (parasite) and host mussel populations, **c** host mussel species regardless their sympatry or local coexistence with *R. ocellatus*. *Values* represent mean and 95% confidence intervals from model estimates (Table 2a), produced in the R package *effects*. An *asterisk* indicates *P* < 0.05
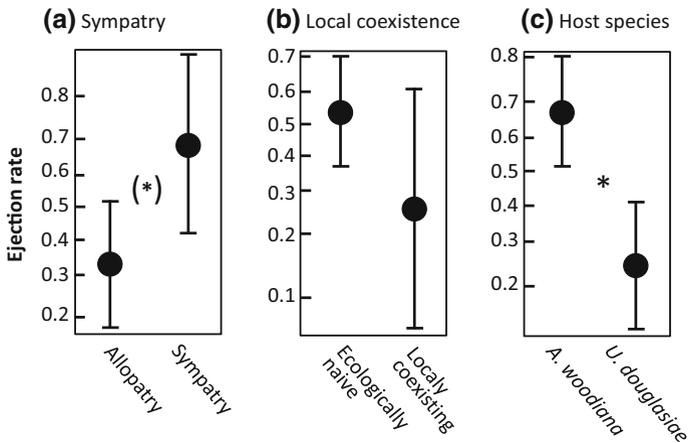
Fig. 3 Mean rate of *R. ocellatus* egg ejection by host mussels with respect to **a** sympatry/allopatry between *R. ocellatus* (parasite) and host mussel populations; **b** local coexistence between *R. ocellatus* (parasite) and host mussel populations; **c** host mussel species regardless their sympatry or local coexistence with *R. ocellatus*. *Values* represent mean and 95% confidence intervals from model estimates (Table 2b), produced in the R package *effects*. An *asterisk* indicate *P* < 0.05, *asterisk in parentheses* indicates *P* ≤ 0.10

(*P* = 0.068). This trend tends to support a role for *host resistance at the meta level*. There was no effect of local host mussel coexistence on egg ejections (Table 2b), discounting the importance of local level effects. The model explained 30% of variability in the data (R^2_{GLMM} marginal = 0.2997), with almost all explained variation related to the fixed part of the model (R^2_{GLMM} conditional = 0.2996).

Discussion

We investigated outcomes in the host–parasite relationship between *R. ocellatus* and its unionid mussel hosts with respect to parasite specialization and host resistance. Using an experimental design that allowed us to distinguish the effects of host–parasite coexistence on a larger geographical level (sympatry/allopatry) and local population level (ecologically relevant local coexistence), we found support for the role of host resistance, with its effects operating at a regional rather than local level. *R. ocellatus* avoided oviposition in sympatric populations of two host mussel species in favour of allopatric populations and there was no effect of local host–parasite coexistence or host species identity on *R. ocellatus* host preference. Ejection of *R. ocellatus* eggs by host mussels was more likely by populations of one species, *A. woodiana*. This host species has higher local population densities and a wider distribution than the other test host species and is, therefore, more likely to be encountered by *R. ocellatus* and other bitterling species. In addition, ejections tended to be higher in sympatric host mussel populations irrespective of host species, providing further indication for the importance of host resistance at a meta level.

Parasite preference for allopatric hosts may interact with host species preference. We found no difference in the preference of *R. ocellatus* between host mussel species, although *A. woodiana* mussels exhibited overall higher egg ejection rates than *U. douglasiae* mussels (Table 2b). The lack of preference for host species may have been overridden by a strong preference for allopatric hosts. *R. ocellatus* is a host generalist, capable of successfully parasitizing both mussel species offered in the study, but demonstrating a certain level of host species preferences (Reichard et al. Reichard et al. 2007a, b). Moreover, conditioning has been shown to affect the choice of host mussel species in the European bitterling *R. amarus*, with a tendency to preferentially exploit host mussel species that were housed with experimental fish (Reichard et al. 2011). In our study, a population of *R. ocellatus* from Nanchang had no previous experience with *U. douglasiae* hosts, but did not exhibit any mussel species preference towards *A. woodiana* hosts. This outcome contradicts the potential role for a conditioning effect and reinforces the importance of generalised host suitability (i.e. the lack of host resistance) in the oviposition-site decisions of *R. ocellatus*. The cues that bitterling use for oviposition decisions appear to include mussel odour, the dissolved oxygen concentration of the water emerging from the mussel and the flow velocity of the exhalant flow (Smith et al. 2001; Mills and Reynolds 2002; Phillips et al. 2017), though these are not yet fully understood. In European unionids, host mussel populations have evolved morphological and behavioural adaptations, including egg ejection, to avoid bitterling oviposition, with mussel populations from areas under stronger parasitic pressure exhibiting enhanced counter-adaptations to reduce parasitism by bitterling (Reichard et al. 2010). It is unclear whether bitterling are capable of perceiving such differences in the level of host counter-adaptation, but our results suggest it may be the case at the host population level, making *R. ocellatus* highly efficient at utilising a broad range of novel host populations and species. However, despite showing appropriate responses, the cues used by *R. ocellatus* to recognise host suitability remain to be identified.

The limited species selectivity by *R. ocellatus* may appear contradictory in the context of host–parasite coevolution, but may reflect an overriding role for host quality traits rather than host specific traits. Thus the dissolved oxygen concentration and flow velocity of the exhalant flow of a mussel may be more reliable predictors of the suitability of a host for the incubation of eggs and embryos than species identity, at least in the case of *R. ocellatus*. This effect arises because host quality, irrespective of species, can change markedly over a

spawning season (Kitamura 2005; Smith 2017). The spawning season of *R. ocellatus* is relatively protracted, starting in April and typically lasting 6 months (Kitamura 2005; Pateman-Jones et al. 2011). As the spawning season advances mussels fill with eggs and embryos and progressively decline in quality as incubation sites. Towards the end of the spawning season bitterling offspring emerge from mussels, which consequently increase in quality as hosts (Kitamura 2005). While we have only tested host mussels without bit-terling eggs and embryos to standardise host condition, the capacity to distinguish the relative quality of an individual mussel when making oviposition-site decisions may be more critical than host species discrimination. Additional studies to compare egg ejection rates among more host species and hosts with varying intensity of infection by *R. ocellatus* would help clarify variation in host preference.

The host mussel choice by *R. ocellatus* was adaptive in terms of the use of host mussel populations that tended to be least effective in ejecting *R. ocellatus* eggs. We have pre-viously detected large differences in capability of European unionid hosts to eject the eggs of the European bitterling, *R. amarus*, between areas of recent and ancient association (Reichard et al. 2010). However, cross resistance experiments between host mussel and fish parasite populations from areas of ancient and recent sympatry were not possible in that study due to the lack of legal approval of animal transport between study regions. The current findings with *R. ocellatus* and their host mussels are consistent with the prediction that naive hosts, evolving in the absence of parasites, relax costly resistance mechanisms in comparison with hosts continuously exposed to parasites. Similarly, relaxed selection by brood parasitic brown-headed cowbirds *Molothrus ater* (Boddaert) on allopatric popula-tions of American robins *Turdus migratorius* L. and gray catbirds *Dumetella carolinensis* (L.) resulted in a lower frequency of ejection of parasite eggs compared with sympatric populations (Kuehn 2009). Analogous observations have been found in other host–parasite systems, such as for the susceptibility of isopod populations to an acanthocephalan parasite (Hasu et al. 2009), and infection by the yeast parasite *Metschnikowia bicuspidata* (Metschnikow) of the crustacean *Daphnia dentifera* Forbes (Auld et al. 2013).

No effect of local coexistence on host mussel preference across *R. ocellatus* populations failed to support the existence of fine-scale local adaptations of *R. ocellatus* to avoid their locally coexisting population of hosts. Parasites are frequently reported to avoid local hosts due to their better resistance, for example in the relationship between the bacterial parasite *Holospora undulata* Hafkine and its protozoan host *Paramecium caudatum* Ehrenberg (Adiba et al. 2010). However, the lack of host–parasite coevolution at a local scale could be explained by other factors. First, studies based on parasites with broad host ranges are less likely to demonstrate local parasite adaptation than those on parasites with narrow host ranges, probably due to evolutionary lags during diffuse coevolution of generalist parasites with their hosts (Lajeunesse and Forbes 2002). In terms of our study system, this would predict more localised coevolutionary dynamics in host specialist bitterling, such as *R. sinensis* (Reichard et al. 2007a). Second, the potential for large-scale dispersal by at least one of the partners mitigates the opportunity of fine-scaled local adaptation. All unionid mussels have a larval stage (glochidium) that attaches to host fish for days to months and may be dispersed over large distances (Dillon 2000). Third, in some systems, local adaptation of the parasite can be observed over a scale of meters (Lively and Jokela 1996), while in other systems parasites exhibit no local adaptation within-regions but do express adaptations at larger distances (Hanks and Denno 1994). Hence, although it is possible that *R. ocellatus* exhibit no local adaptation at the scale tested here, we cannot preclude local adaptation on a larger environmental scale, particularly given the limited number of test populations and the use of naive mussels as one of the host populations.

Understanding the coevolutionary dynamics between *R. ocellatus* and *A. woodiana* has become a pressing question because *A. woodiana* is an invasive species across Europe, Asia, and the Americas (Watters 1997; Douda et al. 2012). The invasive range of *A. woodiana* includes large parts of the range of the European bitterling *R. amarus* (Reichard et al. 2015). While we show that *A. woodiana* was capable of ejecting a large proportion of eggs oviposited by *R. ocellatus* in its gills, its ejection rate of *R. amarus* eggs is even greater (Reichard et al. 2012, 2015). Notably, *R. ocellatus* is itself an invasive species in several Asian countries (Welcomme 1988; Kawamura et al. 2001; Vasil'eva et al. 2015). Our results show a preference for allopatric, evolutionarily naive hosts, regardless of host species identity. In order to establish, persist and spread without their native hosts, introduced parasites must either be generalist species that can infect a large range of new hosts, with at least some degree of success, or specialize on a new host species in the novel communities they invade (Font 2003; Frankel et al. 2015). One of the primary reasons for the invasive success of *R. ocellatus* may be its capacity to exploit a wide variety of mussel species and populations as hosts, coupled with the fitness advantage demonstrated here in using naive host populations due to their lower egg ejection rates (Reichard et al. 2010). Given that several successful parasite invasions have been attributed to the use of naive hosts species owing to the absence of an effective adaptive response (Kelehear et al. 2015), *R. ocellatus* is predicted to successfully reproduce and establish non-native populations in other geographic regions.

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