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PRIMARY RESEARCH PAPER

No effect of recent sympatry with invasive zebra mussel on the oviposition decisions and reproductive success of the bitterling fish, a brood parasite of unionid mussels

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Abstract The presence of non-native species can affect coevolved relationships. However, rapid reciprocal changes in coevolutionary associations provide the potential to quickly respond to a new situation. We studied a system where bitterling fish (*Rhodeus amarus*) parasitize unionid mussels by laying their eggs onto their gills. This association is affected by the infestation of unionid shells by the non-native zebra mussel (*Dreissena polymorpha*). In a series of experiments under experimental, semi-natural and natural conditions, we compared the behavioural response to zebra mussel infestation of unionid shells, its effect on oviposition decisions and their population consequences between bitterling populations naïve to zebra mussels and those recently sympatric with zebra mussels. We found no effect of recent sympatry on bitterling preoviposition behaviour and oviposition decisions and only a weak effect on their reproductive success. Bitterling from both populations inspected infested and non-infested mussels at the same rate but preferred to oviposit into non-infested unionid hosts. However, neither bitterling population completely avoided oviposition into infested unionids and three ovipositions into zebra mussels were observed. Overall, there was a clear negative relationship between the number of zebra mussels on unionid host shells and the number of juvenile bitterling emerging from the mussels. Our study demonstrated a lack of rapid evolutionary response to adaptively modulate oviposition choice after recent zebra mussel invasion.

Keywords Ecological naivety · Population consequences · Rapid adaptation · *Unio* · Unionida

Introduction

Human-mediated range expansions and invasions threaten many aspects of biodiversity, including interspecific ecological relationships (Shine, 2012; Lockwood et al., 2013; Simberloff et al., 2013; Ricciardi et al., 2013). Many interspecific relationships involve coevolution (Kiers et al., 2010). Interacting species often coexist in spatially structured populations that may coevolve at different rates, producing a geographic mosaic of adaptations and counter-adaptations with local coevolutionary hotspots and coldspots (Thompson, 2005). For example, the corolla tube length of the plant *Zaluzianskya*

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microsiphon (Kuntze) and the proboscis length of its main pollinator, a long-tongued fly, *Prosoeca ganglbaueri* Lichtwardt, 1910, vary significantly among sites in strong correspondence (Anderson & Johnson, 2007). Likewise, the level of tetrodotoxin resistance in the garter snake *Thamnophis sirtalis* (Linnaeus, 1758) covaries spatially with the presence of toxic newts of the genus *Taricha.* Where the newts are absent or nontoxic, *T. sirtalis* resistance to tetrodotoxin is minimal (Brodie et al., 2002). These and other examples demonstrate that coevolutionary dynamics can occur at a fine scale.

Human-induced changes to coevolutionary relationships may also be extremely rapid. The shift to a novel host plant species used by a host-specialist butterfly was observed over a period of less than 10 years, following a change in plant community caused by a major human disturbance (Singer et al., 1993). Such rapid disturbances and human-assisted species translocations may have dramatic impact on non-native species, mediated by a shift in coevolutionary state between native and novel partners (Carroll et al., 1998; Prior et al., 2015; Dunphy et al., 2016). For example, a parasite species can spread from a region of long-term sympatry with its host (where it is subject to a coevolutionary arms race of adaptation and counter-adaptation) into a new area with naïve hosts resulting in benefits to the parasite from the coevolutionary lag. A nematode parasite of East Asian eels, *Anguillicoloides crassus* (Kuwahara, Niimi & Hagaki, 1974), introduced to Europe with its native host, Japanese eel, *Anquilla japonica* Temminck & Schlegel, 1846, imported for aquaculture, infected local populations of the European eel, *Anguilla anguilla* (Linnaeus, 1758). This has led to their massive mortality as *A. crassus* was too virulent in the non-coevolved European eel hosts (Taraschewski, 2006). Similar cases often involve long-distance range expansion, with species colonizing habitats with fundamentally different biological communities (Lockwood et al., 2013; Janác et al., 2016), but subsequent range expansions are often incremental (Sousa et al., 2014).

Here, we study the coevolutionary relationship between a freshwater fish, the European bitterling, *Rhodeus amarus* (Bloch, 1782), freshwater unionid mussels (Unionidae) that serve as hosts that brood bitterling embryos, and the invasive zebra mussel *Dreissena polymorpha* (Pallas, 1771). Unionid mussels live in benthic sediment and filter the surrounding water to obtain food and oxygen. Zebra mussels attach to hard substrates and preferentially use the shells of living unionids as attachment substrate (Lewandowski, 1976; Schloesser et al., 1996; Lucy et al., 2014). This fouling of unionid shells is specifically targeted near the siphons that lead into the gill cavity, which interferes with unionid feeding (Pilotto et al., 2016). At the same time, it also decreases the ability of bitterling to parasitize the unionid by depositing their eggs in the mussel gills (zu Ermgassen & Aldridge, 2010; Vrtílek & Reichard, 2012). In a series of experiments under laboratory, semi-natural and natural conditions, we expanded on previous studies (zu Ermgassen & Aldridge, 2010; Vrtílek & Reichard, 2012) and tested whether evolutionary changes following recent sympatry (approximately 10 generations) affected bitterling oviposition decisions and the reproductive consequences of using unionids infested with zebra mussels.

The European bitterling is small fish that reaches sexual maturity after its first winter at a size of 30–35 mm (Reichard & Jurajda, 1999). Its reproduction is dependent on the use of live freshwater mussels as incubation sites for their embryos (Smith et al., 2004). During the reproductive period (typically 6 weeks in spring), males develop bright nuptial coloration and defend territories. Female bitterling cyclically extend long ovipositors as they ovulate a set of eggs. Females with extended ovipositors are courted by males who lead them to unionid mussels in their territories. Fish thoroughly inspect the siphons of potential hosts and make sophisticated host choices based on the host quality (Smith et al., 2000a, 2001; Candolin & Reynolds, 2001). On deciding to spawn, the female inserts her ovipositor into the mussel gills via the exhalant siphon and lays a batch of eggs (Smith et al., 2004). The eggs are fertilized by sperm released over the inhalant siphon of the host, discharged both before and after oviposition (Reichard et al., 2004a). Water filtered by the mussel then carries the sperm through the gills and fertilizes the eggs (Smith et al., 2004).

The European bitterling co-occurs with one to four mussel species (Smith et al., 2004), *Anodonta cygnea* (Linnaeus, 1758), *Anodonta anatina* (Linnaeus, 1758), *Unio pictorum* (Linnaeus, 1758) and *Unio tumidus* Philipsson, 1788, all of which may serve as their hosts (Reynolds et al., 1997). However, bitterling display preferences both among unionid species and among individual mussels (Smith et al., 2000a) that maximize their reproductive success (Smith et al., 2000b; Mills & Reynolds, 2002). Host choice is likely related to oxygen conditions in the mussel gills (Smith et al., 2001) and mussel's ventilation rate (Mills & Reynolds, 2002). Female bitterling often abandon a mussel following inspection when its perceived quality is suboptimal (Spence et al., 2013).

The zebra mussel is a small freshwater bivalve with planktonic larvae that settles on hard sediment and filters food particles from water column. It is one of the most detrimental invasive species (DAISIE, 2016) and causes substantial changes to aquatic ecosystems worldwide due to its high filtration activity (MacIsaac, 1996; Sousa et al., 2009; Strayer, 2009; Higgins & Vander Zanden, 2010; Karatayev et al., 2015). The attachment of zebra mussels to hard substrates causes considerable economic and ecological damage (Strayer, 2009; Sousa et al., 2014; Karatayev et al., 2015), with the impact on native unionids, especially in North America, being probably its most serious direct ecological effect (Strayer, 2009; Lucy et al., 2014). Zebra mussel fouling on other bivalves interferes with the normal function of their siphons, hampers the movement of valves, causes shell deformities, disrupts host stability, locomotion and burrowing ability, and may lead to smothering caused by complete occlusion of the siphons (Mackie, 1991). In addition, zebra mussels directly compete for food with their unionid hosts (Baker & Levinton, 2003; Bódis et al., 2014). Zebra mussel infestation can lead to high unionid mortality (Ricciardi et al., 1996). Settling on a living unionid increases zebra mussel condition (Pilotto et al., 2016). Thus, zebra mussels benefit from their association with unionids, while this association appears to have a consistently negative impact on unionids (e.g. Haag et al., 1993; Baker & Hornbach, 1997; Sousa et al., 2011; Bódis et al., 2014).

The zebra mussel is native to the Ponto-Caspian region (Son, 2007), where it is naturally sympatric with European unionid mussel species (Bauer & Wächtler, 2001; Lopes-Lima et al., 2016) and with the European bitterling (Bohlen et al., 2006; Van Damme et al., 2007; Bryja et al., 2010). It has expanded into large parts of the Northern Hemisphere during the last 200 years (reviewed in Karatayev et al., 2015). This expansion included a substantial part of central and western Europe. It is now associated with local, evolutionarily naive populations of unionid mussels. These unionid populations are frequently exploited by European bitterling. In a study from Great Britain, where both zebra mussels and bitterling are non-native, zu Ermgassen and Aldridge (2010) demonstrated that even a small number of zebra mussels may have negative impact on bitterling reproductive success. Non-infested unionids contained significantly larger numbers of bitterling embryos than zebra mussel infested unionids, regardless of whether the zebra mussels were alive or artificially glued dead shells (zu Ermgassen & Aldridge, 2010). This has been largely confirmed by Vrtílek and Reichard (2012) from the middle Danube basin (Czech Republic), where the European bitterling is native and zebra mussels invading. They found that abundances of below five live zebra mussels per unionid had only negligible impact on the number of bitterling eggs laid to the unionid but the abundances over 10 zebra mussels prevented bitterling from using the unionid host. They suggested that although bitterling often attempted to oviposit into infested unionids, most attempts failed as the ovipositor did not reach the unionid gills (Vrtílek & Reichard, 2012).

Here, we build on previous knowledge and test whether there is any difference in oviposition decisions and their consequences between two adjacent European bitterling populations—one sympatric with zebra mussel for at least 10 generations and a naïve control population. We hypothesized that the experienced bitterling population would suffer a lower cost of zebra mussel infestation due to a rapid evolutionary response or individual experience. To test this hypothesis, we observed the pre-oviposition behaviour and oviposition decisions of male and female bitterling from each population in aquaria. We predicted that bitterling from the population sympatric with the zebra mussel have evolved a lower acceptance of zebra mussel-infected unionid hosts, expressed by a lower host preference behaviours and lower oviposition rate into infected hosts. We then recorded the reproductive success of replicated experimental bitterling populations in a long-term mesocosm study and predicted the negative effect of infestation to be lower in the sympatric bitterling population. For these two experiments, we used five zebra mussels located near the siphon, as it is a common infestation density in natural unionid populations in our study area, and does not mechanically prevent bitterling from using infested unionid hosts, but has the potential to decrease bitterling egg load in unionid gills (Vrtílek & Reichard, 2012). Further, this density does not significantly affect the condition of the unionid host, measured as glycogen reserve (zu Ermgassen & Aldridge, 2010). Therefore, this number of zebra mussels does not compromise host quality and thus a difference between the two bitterling populations could be inferred as a coevolutionary response. To place our results in their natural context, we compared natural bitterling releases from two zebra mussel-infested *Unio* spp. populations (from separate field sites) across a range of hosts with variable zebra mussel infestations. We predicted negative relationship between zebra mussel infestation and bitterling releases from host mussels.

Materials and methods

Study area and source populations

Fish were collected from two populations. The River Kyjovka (48°46′43″N, 17°00′58″E) is devoid of zebra mussels (zebra mussel-naïve population), while Lake Hvězda (48°38′36″N, 16°55′57″E, a borrow pit) contains zebra mussels at least from 2005 (M. Reichard, personal observation), i.e. 10 years (and 10 bitterling generations) prior to the experiments (zebra mussel-sympatric population). The fish were caught by electrofishing on 22 April 2015 (i.e. in the first part of the bitterling reproductive season), transported to the laboratory of the Institute of Vertebrate Biology in Brno and kept in large outdoor pools. This ensured that the sympatric fish had experienced zebra-mussel infested hosts prior to their use in the experiments. Fish were fed daily with frozen chironomid larvae, fish flakes and pellets, and live algae and invertebrate fauna that established in the pools. Unionid mussels were collected by hand from two lakes—Lake Hvězda (the same site as zebra mussel-sympatric bitterling population) and Lake Týnecké (48°44′00.8″N 17°01′02.1″E, oxbow lake). Both sites are part of the River Morava floodplain (that includes the River Kyjovka, a small tributary of the Morava) and are located within a radius of 15 km in the southeastern part of the Czech Republic. However, any recent dispersal of fish and unionid mussels is severely restricted by regulation and channelization of floodplain completed in the 1980s (Jurajda, 1999). The two bitterling populations tested have the same genetic background (Bryja et al., 2010). The source of the zebra mussel invasion is not known but this species was present in the River Morava basin and its spread to several new sites coincided with a large flooding of the entire basin in 1997 (Reichard et al., 2001; Jurajda et al., 2006). All data were collected in spring and summer 2015.

Behavioural experiment

To test the effect of zebra mussel infestation on pre-oviposition behaviour related to host choice and final oviposition decisions of male and female bitterling from populations sympatric and naïve to zebra mussels, a set of 113 L aquaria (75 × 40 × 40 cm) was used. Aquaria were equipped with a layer of sand on the bottom and were continuously aerated. Adjacent aquaria were isolated by opaque barriers to avoid any interaction between fish from neighbouring aquaria. Two size-matched *Unio tumidus* mussels (common hosts of the European bitterling) from Lake Týnecké were placed in the centre of the aquarium, 25 cm apart. One *U. tumidus* was devoid of any zebra mussel; the second *U. tumidus* had five zebra mussels located near the siphons. Non-infested *U. tumidus* typically lacked zebra mussels naturally, but some originally possessed up to 4 zebra mussels that were removed at least one day prior to the experiment. Given that infestation of 5 zebra mussels does not change the condition of their unionid hosts (zu Ermgassen & Aldridge, 2010), it provided a matching condition of the infested and control *U. tumidus.* Experimental *U. tumidus* were collected prior to the beginning of the bitterling reproductive period to ensure that they contained no bitterling eggs or embryos. Each experimental *U. tumidus* was placed in a plastic cup filled with sand to constrain its movement and allowing natural positioning and filtration. The position of infested and control *U. tumidus* within each aquarium was randomly determined.

Single male bitterling in reproductive condition (with intense nuptial colouration) from one population (sympatric or naïve to zebra mussel presence) was placed in the aquarium at least 45 min prior to behavioural observation to enable him to establish a territory. After territorial behaviour was observed, a female in spawning condition (clearly determined by the extended ovipositor) from the same population as the male was placed into the same experimental aquarium, housed in a perforated transparent plastic cup with pierced holes to enable visual and olfactory communication. The female was gently released from the cup after the male started his courtship and behavioural observation began when the first act of courtship towards the free-swimming female was recorded. Behaviour was recorded for a maximum of 20 min but was terminated earlier when oviposition occurred because post-spawning behaviour changes abruptly and is not related to mussel choice.

We recorded the following behaviours (Reichard et al., 2004b): *male leading* the female towards one of the mussels, *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 bitterling embryos), *male sperm release* (indicating investment into a particular host mussel, clearly detected by typical male movement over the mussel inhalant siphon), *female skimming* (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). These behaviours indicate preference for a particular host mussel (Smith et al., 2004).

After completion of each observation, mussels and fish were removed and were not used again in the experiment. We completed a total of 21 observations from the zebra mussel-naïve bitterling population and 17 observations from the zebra mussel-sympatric population. Based on previous research on bitterling oviposition decisions (Smith & Reichard, 2005; Reichard et al., 2007a; Casalini et al., 2013), this was considered a sufficient number of replicates to detect any biologically meaningful differences between populations. The experiment was conducted during April and May 2015, at the peak of the bitterling spawning season (Konečná & Reichard, 2011).

Mesocosm experiment

The effect of zebra mussels on bitterling reproductive success was compared between zebra mussel-naïve and zebra mussel-sympatric populations. This experiment was conducted in outdoor fibreglass tanks (1.3 × 1.3 m, 0.6 m deep) positioned in the garden of the Institute of Vertebrate Biology in Brno, Czech Republic. Tanks were equipped with a layer of sediment and colonized by a natural algal and faunal community. Artificial plants served as refuges for fish in each tank. Four *U. tumidus* mussels were placed in each tank, each in separate plastic cup with sand to keep the mussels in position. Three treatments were imposed—(*A*) four *U. tumidus* infested by zebra mussels; (*B*) two non-infested and two infested *U. tumidus*; and (*C*) four non-infested *U. tumidus* (as a control). The mean (95% confidence interval) level of zebra mussel infestation per mesocosm was 75 (95% confidence interval 64–85) and 19 (95% confidence interval 8–30) and individuals per mesocosm in the *A* and *B* treatments, respectively. At the level of individual unionids, the mean (95% confidence interval) level of zebra mussel infestation per unionid was 20 (95% confidence interval 18–23; full range 8–62) and 11 (95% confidence interval 8–15; full range 5–22) individuals per *U. tumidus* in the *A* and *B* treatments, respectively. Therefore, the treatments *A* and *B* differed in the level of infestation at the mesocosm level and individual *U. tumidus* host level.

In each tank, there were four male and four female bitterling originating from either a zebra mussel-naïve (River Kyjovka fish) or a zebra mussel-sympatric population (Lake Hvězda fish). Six replicates were completed for each treatment, giving a total of 36 experimental populations. The bitterling were allowed to lay their eggs from the end of April to end of June, for a period of 8 weeks. Any juvenile fish that emerged from the mussels (after approximately 4 weeks of incubation) were regularly collected and counted until the beginning of August. There were some instances of female bitterling mortality during the experiment (a maximum of one female per tank). To account for a potential effect on the number of juveniles, we included female mortality as a covariate in the statistical analysis.

Field study

To validate the experimental results observed in captivity, we acquired additional data from natural conditions. We collected unionid mussels from two sites where zebra mussels co-occurred with unionids and bitterling at the second part of bitterling reproductive season on 5 June 2015 (when unionid hosts' infection by bitterling embryos was expected to be highest). We took 48 *Unio tumidus* from Lake Týnecké and 48 *Unio pictorum* from Lake Hvězda. The mussels were collected by hand and transported in aerated water containers into the Institute of Vertebrate Biology in Brno. Mussels were divided into three categories on the basis of zebra mussel infection intensity—(a) non-infested, (b) medium infestation (range of 12–38 zebra mussels), and (c) high infestation (47–123 zebra mussels). The mussels were placed in outdoor fibreglass tanks (as in the mesocosm experiment) and separated into individual mesh bags (mesh size 1 mm). Each bag contained a plastic cylinder (diameter 20 cm) with sediment at the bottom and the top extended above the water surface. Placing the mussels in bags enabled accurate counts of juvenile bitterling emerging from each unionid host as well as insuring standardized developmental conditions. Emerging juvenile bitterling were regularly counted from mid June to the beginning of August.

Data analysis

Data were analysed in R, version 3.2.4 (R Core Development Team, 2014). Behavioural data were analysed using a Generalized Linear Model (GLM) with a Bernoulli distribution applied to female oviposition and a GLM with a negative binomial distribution applied to the counts of male and female mussel inspection events, using *lme4* library (Bates et al., 2014). The fixed effects were unionid infection status (infested or non-infested by zebra mussels) and bitterling population (zebra mussel-naïve or zebra mussel-sympatric). The interaction between infection status and population was also included. Male mussel inspection strongly correlated with male leading behaviour (Spearman correlation: $\sigma = 0.849$, $P < 0.001$) and male sperm releases ($\sigma = 0.790$, $P < 0.001$), and female mussel inspection strongly correlated with female skimming ($\sigma = 0.743$, $P < 0.001$), rendering them redundant in the analysis.

Data on bitterling juveniles were zero-inflated, with 58% (field) and 28% (mesocosm) of mussels (or experimental populations, respectively) releasing no bitterling. Therefore, negative-binomial models (from the *MASS* library) were used to compensate for overdispersion detected in the models with a Poisson distribution (Ripley et al., 2016). Source population (zebra mussel-naïve or zebra mussel-sympatric), zebra mussel infection intensity and the interaction were used as fixed factors. Infection intensity was modelled both as a continuous variable (number of zebra mussels per unionid) and as a categorical variable (A: non-infested, B: medium infestation (12–38 shells), and C: high infestation, >47 shells). Both models provided qualitatively identical outcome but modelling infection intensity as a continuous variable resulted in high overdispersion; we therefore report the model with infestation as a categorical variable.

Results

Behavioural experiment

There was no difference in male inspection behaviour toward infested and non-infested *U. tumidus* (negative binomial GLM; sympatry: $\chi^2 = 1.56$, df = 1, $P = 0.212$; sympatry by infestation interaction: $\chi^2 = 0.02$, df = 1, $P = 0.885$); males from both populations inspected infested and non-infested mussels at the same rate ($\chi^2 = 0.25$, df = 1, 74; $P = 0.616$; Fig. 1a). Testing male leading behaviour or male sperm releases instead of male inspection of mussel siphons gave concordant results.

Likewise, sympatry with zebra mussels had no effect on female inspection behaviour (negative binomial GLM; sympatry: $\chi^2 = 1.96$, df = 1, $P = 0.162$; interaction: $\chi^2 = 0.19$, df = 1, $P = 0.682$) and females paid the same amount of attention to infested and non-infested *U. tumidus* ($\chi^2 = 0.41$, df = 1, $P = 0.521$; Fig. 1b). Testing female skimming behaviour gave concordant results.

Of 38 behavioural observations, 26 resulted in oviposition (13 cases for each bitterling population). There was no effect of bitterling sympatry with zebra mussels on the rate at which the fish oviposited into zebra mussel-infested *U. tumidus*. Fish from each population oviposited 9 times into non-infested *U. tumidus* and 4 times into infested *U. tumidus*. In 3 cases (i.e. 38% of oviposition into infested mussels), the eggs were actually deposited into a zebra mussel rather than the unionid host. Formal tests confirmed that the oviposition rate was higher into non-infected *U. tumidus* (binomial GLM: $\chi^2 = 5.96$, df = 1, $P = 0.015$), regardless of bitterling-zebra mussel sympatry ($\chi^2 = 0.48$, df = 1, $P = 0.489$; interaction: $\chi^2 = 0.12$, df = 1, $P = 0.894$; Fig. 1c).

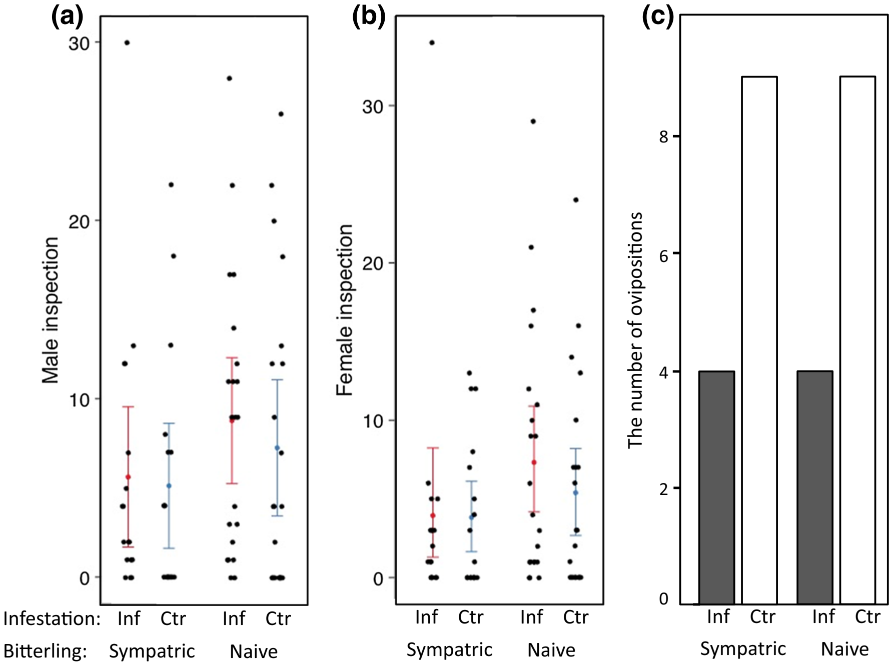

Fig. 1 Preoviposition behaviour and oviposition decisions of bitterling populations sympatric and allopatric to zebra mussels, in a paired choice between *U. tumidus* host infested by five zebra mussels (Inf) and control, uninfested *U. tumidus* (Ctr). **a** The rate of male inspection of host siphons, **b** the rate of female inspection of host siphons, and **c** the number of ovipositions into particular hosts. Observed data with bootstrapped 95% confidence intervals are given for inspection behaviour

Mesocosm experiment

The number of juveniles emerging from experimental mussels strongly declined with increasing proportion of zebra mussel-infested hosts (negative binomial GLM: $\chi^2 = 89.31$, df = 2, $P < 0.001$) and the negative effect of infestation was, unexpectedly, significantly stronger in the sympatric bitterling population (interaction: $\chi^2 = 12.02$, df = 2, $P = 0.003$), although there was no overall difference between bitterling populations in the number of juveniles produced ($\chi^2 = 1.06$, df = 1, $P = 0.304$; Fig. 2). Female mortality during the experiment had no effect on the number of juveniles ($\chi^2 = 0.01$, df = 1, $P = 0.908$) and was excluded from the final model.

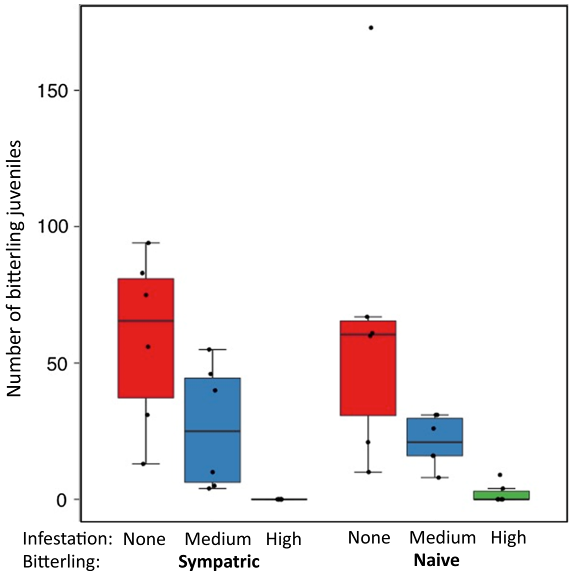

Fig. 2 The effect of zebra mussel infestation of *U. tumidus* on the number of juveniles emerging from hosts in a mesocosm experiment with four uninfested hosts (None), two infested and two non-infested hosts (Medium) and four infested hosts (High) in bitterling populations sympatric and allopatric with zebra mussels. Black circles are observed data

Field data

Infestation by zebra mussels significantly decreased the number of juvenile bitterling emerging from their unionid hosts (negative binomial GLM: $\chi^2 = 43.94$, df = 2, $P < 0.001$; Fig. 3). The number of juveniles emerging differed between two bitterling populations ($\chi^2 = 7.32$, df = 1, $P = 0.007$). This was a consequence of natural difference in infestation intensity between the two populations, as there was no interaction between population identity and zebra mussel infestation intensity ($\chi^2 = 3.68$, df = 2, $P = 0.159$). Modelling the effect of infection intensity as a continuous variable (number of zebra mussels per unionid) gave concordant results (Fig. 4).

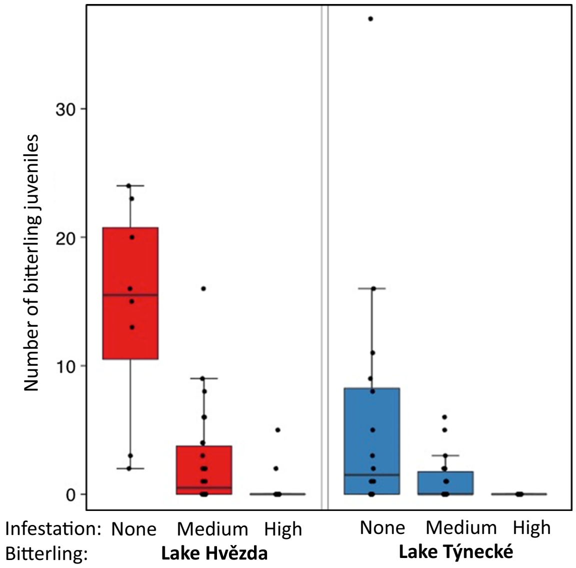

Fig. 3 The effect of natural zebra mussel infestation of *U. tumidus* (Lake Hvězda) and *U. tumidus* (Lake Týnecké) on the number of juveniles emerging from hosts. Zebra mussel infestation was pooled into three categories, with non-infested and weakly infested hosts (with zebra mussels not interfering with unionid siphons; None), intermediate host infestation (Medium) and high host infestation (High). Black circles are observed data

Discussion

Within the framework of rapid coevolutionary responses to the impact of non-native species on established interspecific associations, we studied how zebra mussel infestation of a unionid host affects the oviposition decisions and reproduction success of the bitterling fish. We compared the response and impact of bitterling populations naïve to zebra mussels or sympatric with them for approximately 10 generations. We found no effect of recent sympatry on bitterling preoviposition behaviour and oviposition decisions and only a weak effect on bitterling reproductive success. Bitterling from both populations inspected infested and non-infested mussels at the same rate, preferred to oviposit into non-infested unionid hosts but did not avoid infested unionid hosts completely. There was a clear negative relationship between the number of zebra mussels on unionid host shells and bitterling juveniles emerging from the mussels. Unexpectedly, this relationship was stronger in the sympatric bitterling population.

Biological invasions and range expansions can be viewed as natural experiments on species interactions. Previous research showed that infestation of five zebra mussels did not affect the preoviposition behaviour of bitterling naïve to zebra mussels (Vrtílek & Reichard, 2012). We demonstrate that the same response is shown by bitterling sympatric with the zebra mussel for approximately 10 generations. This implies no rapid evolutionary response by the bitterling and no role of previous individual experience, as the fish used in the experiment were wild-caught and sympatric-population fish were exposed to zebra mussel-infested unionids prior to the experiments. In a conceptually matching case, mosquito fish, *Gambusia holbrooki* Girard, 1859, sympatric to cane toads, *Rhinella marina* (Linnaeus, 1758), ignored toad tadpoles (a toxic prey) while toad-allopatric mosquito fish consumed some tadpoles initially and rapidly developed an aversion (Wijethunga et al., 2016). The experiments then revealed that coevolution (innate effect) and experience (learning effect) both contributed to toad-tadpole aversion with a short period (<100 years) following the colonization of Australia. Other cases of human-mediated shift in host use can take as short time as less than 10 years. Two populations of a host specialist butterfly from North America, the checkerspot, *Euphydryas editha* (Boisduval, 1852), rapidly adapted to new exotic hosts associated with human disturbance of their meadow habitats (Singer et al., 1993). In fish, experimental evolution demonstrated that rapid evolutionary response can be detected as soon as after two generations when selection is strong (Kotrschal et al., 2013).

In our study, sympatric and naïve fish inspected infested and non-infested unionids at the same rate, although their actual oviposition decisions were strongly biased toward non-infested hosts. It is likely that their innate ability to detect mussel quality and make sophisticated oviposition decisions, on the basis of multiple cues (Smith et al., 2001; Mills & Reynolds, 2002; Mills et al., 2005; Reichard et al., 2007b), have overridden any learned or evolutionary response to zebra mussels leading to immediate refusal of zebra mussel-infested host. Bitterling and zebra mussels co-occur naturally in the Pontic region, the eastern part of the European bitterling range (Bohlen et al., 2006; Bryja et al., 2010) and bitterling are commonly associated with zebra mussel infested unionids in some lakes in Turkey (Reichard et al., 2010; Ercan

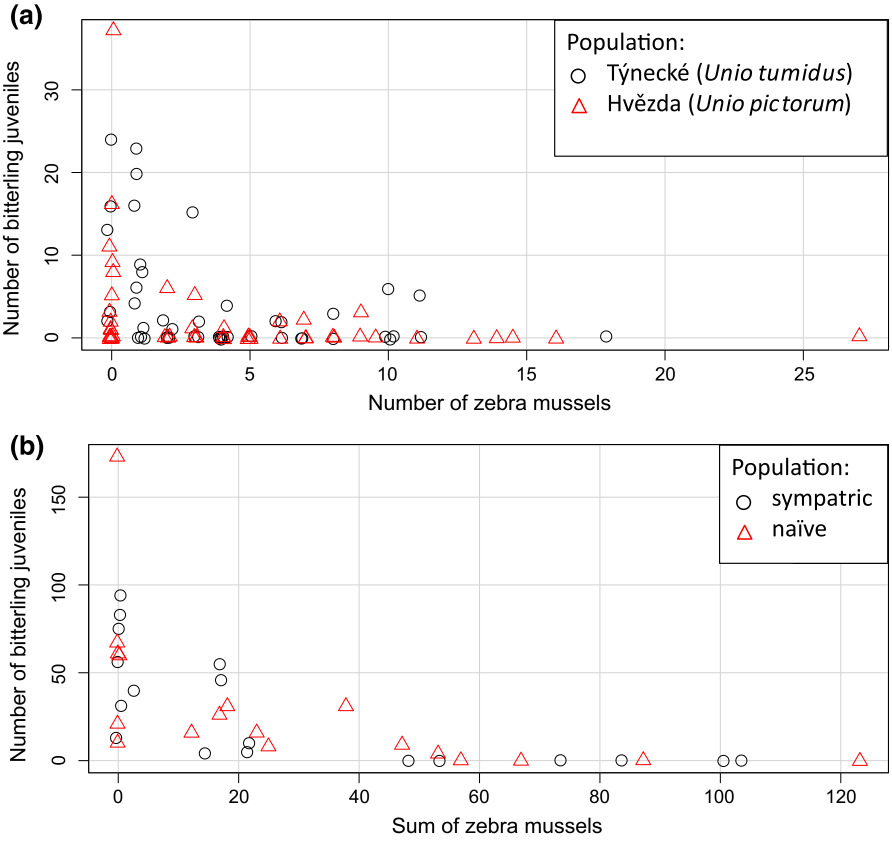

Fig. 4 Bivariate plot of the number of juvenile bitterling emerging from host mussels and the level of their zebra mussel infestation in natural populations (**a**) and in a mesocosm experiment (**b**). Note that it was not possible to assign individual bitterling to individual unionid hosts in the mesocosm experiment and the cumulative number of zebra mussels across four unionid hosts is given

et al., 2013; M. Reichard unpublished data). Further, the zebra mussel (and European bitterling) range may be expanding and contracting cyclically with Quaternary climatic fluctuations (Van Damme et al., 2007; Bryja et al., 2010) and bitterling populations may have been exposed to zebra mussels in their evolutionary past. It would be interesting to compare the response to zebra mussel-infested hosts with bitterling populations in long-term sympatry with zebra mussels (e.g. along Black Sea coast of Turkey) to test whether much longer exposure to zebra mussels is needed to produce a coevolutionary response from bitterling or bitterling populations across Europe possess pre-adapted responses to zebra mussel-infested unionid hosts.

Host inspection behaviour of the bitterling is likely costly. It often takes several minutes for fish to make a decision to use a particular mussel or to continue searching for a more suitable host (Smith et al., 2004). During host inspection, male and female place themselves near the mussel siphons, with their bodies positioned at an angle of approximately 75°, and examine the water coming from the mussel gills. During that phase, fish are certainly more prone to predation. Inspection behaviour is also costly in terms of time spent inspecting unsuitable hosts, which prolongs the overall time devoted to reproductive behaviour. With 4 to 15 oviposition acts during a single day (Smith et al., 2004), each typically lasting 2 to 15 min, a protracted period of inspection is likely non-trivial. Females spend most time grazing algae during the reproductive season to maximize their egg production (Przybylski, 1996).

Inspection of a potential unionid host infested with zebra mussels may, however, be also potentially beneficial. Given that embryo mortality inside the mussel gills is density-dependent (Smith et al., 2000a), the use of zebra mussel-infested hosts might confer a fitness benefit if it contains fewer bitterling embryos. In some circumstances, the risk of oviposition failure or lower oxygen availability to bitterling embryos due to zebra mussel infestation may be lower than the cost of density dependent embryo mortality risk in non-infested hosts. The quality of unionid hosts in terms of their suitability for the bitterling is known to be seasonally dynamic (Smith et al., 2000b; Kitamura, 2006; Smith, 2017) but this hypothesis requires further study.

For the behavioural experiment, we used a moderate density of zebra mussels (5 individuals), which is a level frequently encountered in the field (zu Ermgassen & Aldridge, 2010; M. Reichard, personal observation). At this density, zebra mussels do not decrease unionid condition (measured as glycogen content) (zu Ermgassen & Aldridge, 2010) and unionid host is able to receive and incubate bitterling embryos (Vrtílek & Reichard, 2012). This is confirmed by our field data, where the number of bitterling juveniles declined steeply with the zebra mussel infection intensity, with 1–2 zebra mussels having a negligible effect and unionids with up to 11 zebra mussels containing some successfully developed bitterling.

Bitterling from the population naïve to zebra mussels had non-zero reproductive success in 2 out of 6 experimental mesocosms where all four unionid hosts were heavily infested by zebra mussels (cumulative load of 47 and 53 zebra mussels), producing 9 and 4 juveniles, respectively. All experimental populations produced some juveniles in the intermediate infestation treatment (combining 2 uninfected *U. tumidus* and two *U. tumidus* with 5–22 zebra mussels), though their numbers were more than 50% lower than the non-infested treatment. This indicates a substantial cost to bitterling reproductive success at the population level.

Unionid mussels in large parts of Europe face parasitation from both bitterling and zebra mussels. It is plausible that low intensities of zebra mussel infestation may afford unionids some protection against significant parasitism by bitterling. Bitterling embryos inside the gills of unionids decrease their growth rate throughout the growing season (Reichard et al., 2006), likely due to disruption of water flow in the gills limiting food intake (Spence & Smith, 2013), competition for oxygen (Smith et al., 2001) and direct mechanical damage to gill epithelium (Stadnichenko & Stadnichenko, 1980). The lower bitterling embryo load in zebra mussel infested unionids has been confirmed in three separate studies (zu Ermgasssen & Aldridge, 2010; Vrtílek & Reichard, 2012; this study) and unionid condition is not affected at low levels of zebra mussel infestation (zu Ermgassen & Aldridge, 2010; Bódis et al., 2014). On the other hand, unionids may suffer significant mortality in the case of intense fouling from zebra mussels and Ricciardi et al. (1996) demonstrated up to 100% unionid mortality in sites with high densities of zebra mussels. Unlike marine bivalves, unionid mussels have evolved in the absence of dominant fouling organisms such as zebra mussels and may be particularly sensitive to such infestation (Ricciardi et al., 1996). Shell fouling limits the filtration ability of unionids and decreases their energy intake, ultimately leading to their mortality (Sousa et al., 2011, but see Bódis et al., 2014). In conclusion, zebra mussels are certainly a greater threat to unionids than are bitterling, despite potentially offering some protection from bitterling parasitism under certain circumstances.

We observed bitterling ovipositing into a zebra mussel three times during the experiment (both zebra mussel-naïve and sympatric fish), representing over one third of all oviposition attempts into infested unionid hosts. Bitterling have never been observed to use zebra mussels as hosts (Balon, 1962; zu Ermgassen & Aldridge, 2010) and they are incompatible—bitterling eggs cannot be fertilized in zebra mussels (Smith et al., 2004). We think that the presence of zebra mussels near the siphon of the unionid hosts and disruption of water flow near infested host may confuse the cues females use to position their ovipositor properly into the exhalant siphon of the unionid. Alternatively, the ovipositor may be mechanically distorted during insertion into the exhalant siphon by the physical presence of zebra mussel shells (Vrtílek & Reichard, 2012). Irrespective of the mechanism, oviposition into a zebra mussel constitutes a large fitness cost to the bitterling. Vrtílek and Reichard (2012) considered that mortality related to inaccurate oviposition could result in very strong selection for avoidance of infested unionids or for optimal use of infested hosts by learning from previous oviposition attempts. However, we have found no support for this hypothesis at the behavioural level, with no difference in behaviour and oviposition choice between sympatric and naïve bitterling fish after 10 generations. At the population level, decrease in juvenile bitterling production was lower in zebra mussel-naïve fish and fish from the naïve population produced some juveniles at the highest densities of zebra mussel infestation. This could potentially indicate that the sympatric fish are more likely to avoid infested mussels over a longer time interval than during brief behavioural experiment. The nature of the mesocosm experiment, however, did not permit to separate the role of oviposition preference and selective bitterling embryo mortality. Finally, if individual learning rather than rapid evolutionary response was stronger driver of the oviposition preference, zebra mussel-naïve fish had sufficient time to modify their oviposition preferences according to their previous experience. Each fish participated in multiple ovipositions over the course of mesocosm experiment. Female bitterling ovulate the eggs approximately every 5–7 days during the reproductive season and lay them in several clutches laid over the course of the day (Reichard et al., 2009). Consequently, each female deposited her eggs at least 20 times during the mesocosm experiment. An experimental design that includes temporal aspects of individual preferences and decisions is needed to test the role of learning.

Non-native species (or population) can affect coevolved relationships, including host–parasite interactions. A new species can serve as an alternative partner in mutualistic and antagonistic associations (Tanaka et al., 2007; Jahner et al., 2011) or alter the current coevolutionary state in the association (Kiers et al., 2010; Dunphy et al., 2016). Here, we demonstrated that parasitism by the zebra mussel largely compromised the ability of the bitterling to use unionid mussel hosts. There appears to be a clear net cost to both native taxa. The unionids suffer a high cost of zebra mussel fouling from direct competition and mechanical problems associated with zebra mussel attachment (Mackie, 1991; Baker & Levinton, 2003). The bitterling fish is prevented from proper use of the host for its embryos. Despite the negative impact on both partners in this association and the high density of zebra mussels in our study sites, neither unionid nor bitterling populations have suffered any dramatic decline in approximately 10 years following the introduction of zebra mussels. In both study sites where bitterling, native unionids (*U. tumidus*, *U. pictorum* and *Anodonta anatina*) and zebra mussels are sympatric, zebra mussel populations exhibit large fluctuations (cf. Simberloff & Gibbons, 2004) but typically colonize more than 80% of the unionids (Bódis et al., 2014; M. Reichard, personal observation). The recent arrival of another invasive bivalve, *Anodonta (Sinanodonta) woodiana* (Lea, 1834) from East Asia (Watters, 1997; Douda et al., 2012) that has quickly become a dominant unionid in European bivalve communities (Lajtner & Crncan, 2011), makes native unionid populations in Europe even more vulnerable, calling for a close monitoring of their status (Lopes-Lima et al., 2016) to avoid the fate of sharply declining North American native unionids (Strayer et al., 2004).

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