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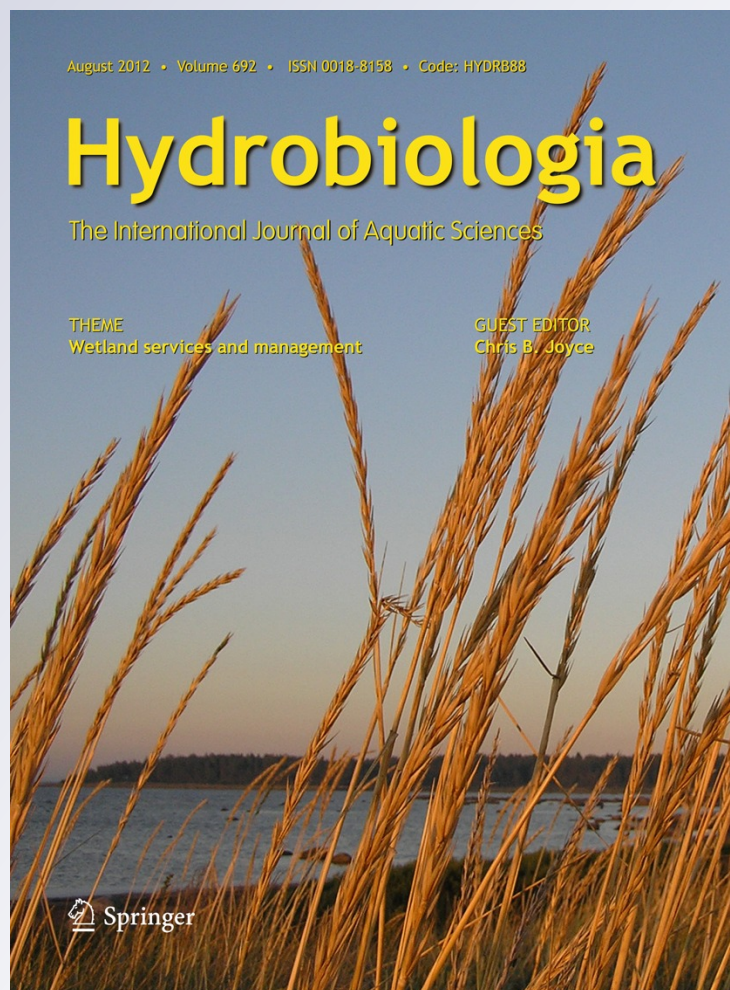
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An indirect effect of biological invasions: the effect of zebra mussel fouling on parasitisation of unionid mussels by bitterling fish

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Abstract Invasive species represent a major threat with both direct and indirect effects on natural ecosystems, including effects on established and coevolved relationships. In a series of experiments, we examined how the interaction between two native species, a unionid mussel (*Unio pictorum*) and the European bitterling (*Rhodeus amarus*), a fish that parasitises unionids, was affected by the non-native zebra mussel (*Dreissena polymorpha*). The zebra mussel fouls hard substrates, including shells of living unionids, and its presence is often associated with a decrease in population density of native unionid mussels. Bitterling lay their eggs into live unionids and the embryos develop inside their gills. Using a range of zebra mussel densities, we demonstrated that zebra mussel fouling had a negative effect on the number of bitterling eggs inside the mussel host, with abundances of 5–10 zebra mussels (shell size 15–25 mm) per unionid critical for bitterling ability to utilise the host. In a further experiment, we found that bitterling did not discriminate between unfouled unionids and those fouled with five zebra mussels. Most ovipositions into fouled hosts, however, were

unsuccessful as eggs failed to reach the unionid gills. We discuss implications of such unsuccessful ovipositions for bitterling recruitment and population dynamics.

Keywords Non-native species · Coevolution · Invasional meltdown · Host–parasite relationship · Aquatic ecosystems

Introduction

The effects of the introduction and establishment of alien species can be precipitated via a multitude of direct and indirect factors (Lockwood et al., 2007), and hence they represent a major threat to natural populations and ecosystems. While interspecific competition or predation represent the most obvious cases of negative impacts, the effects are often indirect and more subtle (reviewed by Simberloff & Von Holle, 1999). For example, an introduction of North American crayfish species to Europe during the second half of the nineteenth century resulted in local extinctions of European crayfish, such as *Astacus astacus* (L., 1758), due to fungal infection (*Aphanomyces astacii*; Schikora, 1903). The American species acts as a vector for the fungus, which is lethal to European crayfish species (Söderhäll & Cerenius, 1999). The benthivorous common carp (*Cyprinus carpio*, L., 1758), an invasive fish species in many parts of the world, stirs up benthic sediment during feeding,

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thereby releasing nutrients into the water column. This can subsequently affect entire ecosystems through interactions at several trophic levels (Wahl et al., 2011). This illustrates a possibility for a non-native species to facilitate establishment of other non-native species, further altering biological community and ecosystem functioning in a process termed invasional meltdown (Simberloff & Von Holle, 1999). Alien species may also influence established and coevolved relationships between native species (Reichard et al., 2012). Here, we examine how interaction between a native fish that parasitizes unionid mussels is affected by the non-native zebra mussel (*Dreissena polymorpha* (Pallas, 1771); Bivalvia: Dreissenidae).

The zebra mussel is considered one of the most damaging of invasive species (Karatayev et al., 1997; Connelly et al., 2007; Ward & Ricciardi, 2007). It has recently expanded its range to many locations outside of its native Ponto-Caspian range (which includes the Caspian, Black and Azov Sea basins (Son, 2007)), including large parts of Europe (DAISIE, 2011) and North America (Strayer, 2009). Zebra mussel invasion is facilitated by the absence of an ecological equivalent in freshwaters, its enormous reproductive ability, a planktonic larval stage (veliger) capable of effective dispersal, and high tolerance of environmental factors (reviewed by Karatayev et al., 1998). The zebra mussel's efficient filtering capacity and its rapid increase in population density in the initial phase of invasion (Mackie, 1991; Strayer & Smith, 1996; Burlakova et al., 2000) has led to intensive nutrient withdrawal from pelagic to benthic systems (Gergs et al., 2009). This is associated with a cascade of ecosystem changes, resulting in a clear water stage (Fahnenstiel et al., 1995) that boosts the growth of macrophytes (Zhu et al., 2006) and may promote fish assemblages dominated by littoral species (Strayer et al., 2004).

Unionid mussels (family Unionidae), including *Unio pictorum* (L., 1758), a species widely distributed in Europe and the preferred host of the European bitterling (*Rhodeus amarus* (Bloch, 1782); Cyprinidae, Acheilognathinae) (Smith et al. 2004), are often negatively affected by zebra mussels (Mackie, 1991; Hunter & Bailey, 1992; Strayer & Smith, 1996; Karatayev et al., 1997; Sousa et al., 2011), resulting in a decrease in population size. Zebra mussels compete with unionids for food particles (Baker & Hornbach, 2000) and also deform their shells via fouling

(Schloesser et al., 1996). Hard substrata (e.g. rocks, gravel and fragments of shells), one of the essential requirements for zebra mussel settling, can be limited in many freshwater systems and unionid shells are frequently used for zebra mussel attachment. Indeed, shells of live unionids appear to be used preferentially, even when there are other hard substrata present (Lewandowski, 1976; Schloesser et al., 1996). Zebra mussels prefer to attach in close proximity to the unionid siphons and, as zebra mussels are efficient and choosy filtrators (Baker & Levinton, 2003), they cause starvation and loss of energetic reserves in the fouled unionids (Baker & Hornbach, 2000; Sousa et al., 2011). Moreover, the fouled unionid inhales metabolic waste and indigestible particles rejected by the zebra mussel. Fouling by zebra mussels is also associated with locomotion impairment and interference in valve movement (Ricciardi et al., 1996).

The bitterling is a freshwater fish adapted to use unionid gills as an exclusive spawning substrate and shelter for their embryos. Male bitterling attract females to live unionid mussels, whereupon females lay their eggs through an exhalant siphon using a long ovipositor. Bitterling are choosy about oviposition site and inspect potential hosts and express host preferences based on mussel quality (Smith et al., 2001, 2004). Oviposition is accompanied by male ejaculation over the inhalant siphon of the unionid, so that the mussel inhales the sperm and fertilisation occurs in the mussel's gills. The embryos then develop inside the mussel for approximately 1 month and leave as free-swimming juveniles (Aldridge, 1999). Bitterling embryos compete with their host for food and oxygen (Spence & Smith, 2012), may block water tubes in the mussel's gills, and damage the epithelium (Stadnichenko & Stadnichenko, 1980), resulting in a reduction in growth rate for the unionid (Reichard et al., 2006).

All three species (unionids, bitterling and zebra mussels) naturally co-occur across a large part of their range in the Ponto-Caspian region. *Unio pictorum* has a naturally wide distribution across much of Europe (Bauer and Wächtler 2001); while bitterling are of East Asian origin (Okazaki et al., 2001), with a single species (sometimes referred to as a species complex) inhabiting Europe from the late Tertiary (Bohlen et al., 2006). Bitterling range fluctuated during the Quaternary and populations over most of Central and Western Europe are likely to be recent invaders, with

arrival from the Ponto-Caspian region millennia or centuries before the present (Kozhara et al., 2007; Van Damme et al., 2007; Bryja et al., 2010). In our study region (Czech Republic), zebra mussels are considered a non-native species, while *U. pictorum* and the European bitterling are considered native species, despite potentially recent bitterling colonisation of Central Europe. The situation varies across Europe, however, from all three species being native in parts of the Ponto-Caspian region to both bitterling and zebra mussel being non-native in western and northern parts of Europe (zu Ermgassen & Aldridge, 2010).

Previous research has shown that bitterling egg load in unionids can be affected by zebra mussel fouling of unionid shells, with a significant reduction in egg load in unionids fouled by zebra mussel compared to non-fouled controls (zu Ermgassen & Aldridge, 2010). The physical presence of zebra mussel shells (alive or dead) resulted in a lower bitterling prevalence (proportion of unionids infected by bitterling eggs and embryos), while there was no difference in bitterling prevalence between unfouled unionids and unionids with their zebra mussels removed prior to experiment (zu Ermgassen & Aldridge, 2010). In addition, while the physiological condition of unionids (measured as glycogen content) was not affected by zebra mussel fouling (zu Ermgassen & Aldridge, 2010) at low and intermediate infestations (five zebra mussels); physiology was significantly affected by high intensity of zebra mussel infestation, which resulted in a decrease in ventilation rate that persisted even after zebra mussel removal (Baker & Hornbach, 2000).

In this study, we examine how zebra mussel fouling of unionids affects oviposition decisions of bitterling using an intensity of infestation regularly recorded at our study sites in the Czech Republic (levels comparable to those reported by zu Ermgassen & Aldridge (2010)). Unlike zu Ermgassen & Aldridge (2010), who focused on bitterling egg abundance associated with zebra mussel fouling under natural and semi-natural conditions in Britain, we concentrate on bitterling reproductive decisions and host choice under experimental conditions. We tested whether bitterling used unfouled unionids preferentially and studied mechanisms for this preference (male and female response at various stages of pre-oviposition decision). We hypothesised that a decrease in bitterling prevalence observed in fouled unionids by zu Ermgassen &

Aldridge (2010) was caused by active preference of adult bitterling for unfouled unionids. This may be because oviposition into a unionid fouled by zebra mussel is physically difficult or that the cues used by bitterling for oviposition decision (quality of water leaving the unionids exhalant siphon in terms of oxygen level, metabolic waste content, and shape and speed of the water current) may be affected. This preference is likely to be adaptive, as bitterling embryos developing in fouled unionids are expected to suffer suboptimal conditions. Specifically, we used two experiments to test (1) whether zebra mussel fouling affects the number of bitterling eggs received by a unionid host and, (2) whether bitterling males or females modify their behaviour in response to low level infestation (five individuals; i.e. the intensity at which unionid physiology is unaffected) of unionid hosts by zebra mussels.

Materials and methods

Experiment 1 (mesocosm study)

This experiment was designed to test how zebra mussel fouling of unionid hosts affects the number of bitterling eggs deposited into a unionid mussel's gills. *Unio pictorum* was used in all experiments as the species is abundant at our study sites (Smith et al., 2000a), is preferentially used by bitterling for oviposition (Smith et al., 2000b), and has the lowest rejection rate of bitterling eggs (Mills & Reynolds, 2002). The same species was also used in the study of zu Ermgassen & Aldridge (2010), allowing comparison of our results.

The experiment consisted of two parts. In the first, intensity of fouling was varied across a broad range of zebra mussel densities (0–43 individuals). In the second, based on the results for presence of bitterling eggs in fouled mussels from the first part of the study, we concentrated on a narrower range of zebra mussel densities (0–9 individuals) lying within the range of steepest decline in bitterling egg load in relation to intensity of zebra mussel infestation. The experiment was conducted in large fibreglass tubs (130 × 130 cm) filled with aged tap water to a depth of 70 cm. The bottom was covered with a layer of gravel and each tub had five plastic pots containing fine sand where

individual *U. pictorum* were kept in a fixed position. This allowed each *U. pictorum* to perform standard movements and normal filtration while preventing complete burial into the sediment. Both fouled and unfouled *U. pictorum* were collected by hand from an oxbow lake (48°44'02"N; 17°01'03"E) near the River Morava prior to onset of the bitterling reproductive season, thereby ensuring that they contained no bitterling eggs. Each tub had a population of bitterling consisting of 5 males and 23 females; a female-biased sex ratio being used to maximise oviposition rate. The bitterling were captured by electrofishing from the River Kyjovka (48°46'44"N; 17°01'00"E), a tributary of the River Morava.

For the first part of the experiment, five *U. pictorum* were used in each tub. One *U. pictorum* was always unfouled, while the other four had fouling intensities in four different categories, i.e. low (4–7 zebra mussel individuals), medium (8–12 inds), high (14–25 inds) and very high (29–43 inds). Unfouled unionids had not been previously infested by zebra mussels in the wild prior to collection, as indicated by an absence of zebra mussel byssal fibres or dead shells. In order to provide *U. pictorum* with a lower intensity of infestation, some zebra mussel individuals were artificially removed from heavily fouled *U. pictorum* shells to required level. This test was completed in six experimental tubs, with fish allowed to oviposit from 23 to 29 May 2008 (7 days). Subsequently, in the second part of this experiment, which took place between 29 May and 4 June (7 days), only four *U. pictorum* fouled by 0–9 zebra mussels were placed in each tub, with the aim to gradually vary zebra mussel infection across the set range. Experimental conditions were identical, though only four experimental tubs were used due to the limited number of experimental fish available.

After each part of Experiment 1, the mussels were collected from the tubs and the attached zebra mussels removed, counted and measured for shell length. The *U. pictorum* shells were then measured along their longest axis and dissected to allow the number of bitterling eggs and embryos to be counted. In both parts of the experiment there was a single tub in which bitterling failed to reproduce over the experimental treatment period (7 days). In these cases, it is likely that no bitterling female came to reproductive condition and hence no oviposition occurred. These tubs were excluded from further analysis.

Experiment 2 (behavioural observation)

This experiment aimed to test whether zebra mussel fouling of *U. pictorum* hosts affects oviposition decisions and pre-oviposition behaviour of male and female bitterling. The experiment followed a paired design, with fish having a simultaneous choice between an unfouled unionid and a unionid fouled by five zebra mussels. Observations were conducted in 24 Iaquaria with a layer of sand on the bottom and two plastic pots with fine sand positioned in the centre of the tank. Each plastic pot contained a single *U. pictorum*, with the position of fouled and unfouled mussel randomised (left, right). All fouled *U. pictorum* possessed five zebra mussels on their shells. We targeted *U. pictorum* naturally fouled by five zebra mussels, though some were originally more heavily fouled and zebra mussel density was artificially reduced to meet the criteria. In each case, attention was paid to standardising (a) the size of zebra mussels, (b) their position on the *U. pictorum* shell and (c) their orientation towards the siphon. Based on the outcome of Experiment 1, a fouling intensity of five zebra mussels was chosen as it represented the intensity at which a negative effect on bitterling egg density could be detected (though the effect was not strong). Mean shell length (\pm SE) of zebra mussels in Experiment 2 was 25.0 (\pm 0.8) mm. Unionids were collected on 6 April 2009 (prior to the bitterling reproductive season), at the same site as for Experiment 1, and held in large outdoor tubs before the start of the experiment. Twenty-one replicates were conducted over 7 days (from 27 May to 2 June 2009).

Unionids and male bitterling in breeding condition (intense nuptial colouration) were placed into the experimental tank. A female in reproductive condition (with a long transparent ovipositor indicating that a batch of eggs had been ovulated) was added to the aquarium in a 0.5-l perforated transparent plastic cup that allowed visual and olfactory communication between partners in order to initiate male courtship. After the male began to court the female, the female was gently released from the cup. Behavioural recording started after the first behavioural act of the fish directed towards the *U. pictorum* (typically male inspection) and lasted for 20 min or until oviposition.

Recorded reproductive behaviour followed definitions by Smith et al. (2004) and consisted of the following categories: *male and female inspection a*

fish positions itself at a 75° angle towards the host mussel siphon, with its snout close to the exhalant siphon; *leading* male swims between a female and a potential host, undulating his body and attracting the female towards the mussel; *skimming* female performs a swinging movement over the mussel but does not insert her ovipositor into the exhalant siphon of the mussel and no eggs are laid; *sperm release* male performs a swinging movement over the inhalant siphon of the mussel and releases sperm, sometimes visible as a whitish cloud; and *oviposition* characterised by female movement over the mussel, including insertion of the ovipositor into the exhalant siphon of the mussel where the eggs are laid. After the end of each observation, fish and mussels were replaced and were not used again in the experiment. Zebra mussels were removed from unionid shells and measured to the nearest 1 mm; and unionids were measured along their longest axis.

Data analysis

Data from the Experiment 1 were analysed using the Generalised Linear Mixed Models (GLMM) in R version 2.13.1 (2011-07-08). The response variable (number of eggs in each *U. pictorum*) followed a Poisson distribution (count data) and, therefore, we used Poisson error structure (*lme4* package (Bates et al., 2011)). Fixed factors were represented by number of zebra mussel individuals on the *U. pictorum* host, *U. pictorum* shell size and sum of zebra mussel shell sizes. We also included a random factor ('tub identity') to account for non-independence of multiple hosts tested with the same population of fish. Stepwise backward deletion of non-significant predictors was applied to build a Minimal Adequate Model, based on the Akaike Information Criterion. Each simplified model was tested against the full model using the log-likelihood test to ensure that dropping of a non-significant term did not reduce the proportion of explained variability (Crawley, 2007). Levels of statistical non-significance for the removed terms were taken from the full model. We tested each part of the experiment separately in order to account for temporal effects due to performing each part of the experiment at a different stage of the season and the repeated use of some fish. Differences in oviposition events between fouled and unfouled unionid hosts in Experiment 2 were tested by exact binomial test.

Behavioural data were not normally distributed and did not respond to transformations; therefore, data were tested using non-parametric Wilcoxon paired tests (Statistica 9.1 for Windows; Statsoft Inc.).

Results

The number of bitterling eggs deposited in unionid gills was negatively related to intensity of zebra mussel fouling. The effect was especially pronounced when a wide range of infestation intensities was used (GLMM with Poisson distribution, $z = -7.41$, $P < 0.001$; Fig. 1a; mean \pm SE zebra mussel shell size: 15.6 ± 0.2 mm). The effects of additional host variables did not explain any further significant

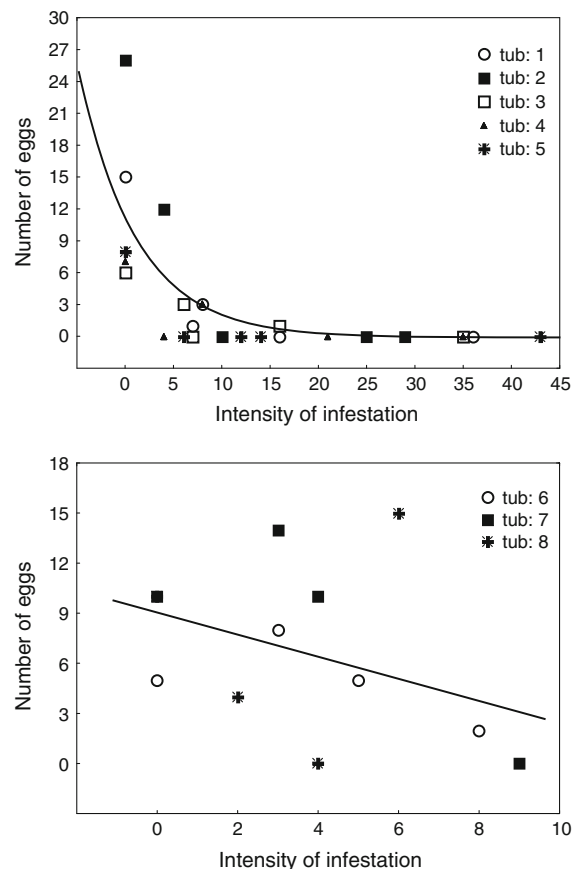


Fig. 1 Relationship between bitterling egg load and intensity of zebra mussel infestation of *U. pictorum* hosts using a wide (a) and narrow (b) range of zebra mussel fouling. Data points for each experimental population are denoted by a different symbol. Curves are fitted using the exponential function $y = 10.32 * \exp(-0.17 * x)$ (a) and the linear function $y = 9.12 - 0.60 * x$ (b)

proportion of variance (unionid host shell length: $z = -0.79$, $P = 0.432$; sum of zebra mussel shell lengths: $z = -0.30$, $P = 0.764$). The negative relationship between number of eggs and intensity of infestation was still observed when a narrower interval of infestation intensity was used (GLMM with Poisson distribution, $z = -3.27$, $P = 0.001$; mean \pm SE zebra mussel shell size: 17.4 ± 0.9 mm). In addition, size of bitterling egg load was positively related to shell length of the *U. pictorum* host ($z = 3.79$, $P = 0.0002$). The sum of zebra mussel shell lengths was not a significant predictor of bitterling egg load ($z = 0.83$, $P = 0.406$). Maximum infestation intensity of *U. pictorum* with at least one bitterling egg was 16 zebra mussels (mean \pm SE zebra mussel shell size: 14.4 ± 0.8 mm). The steepest decline in bitterling egg number in the gills of *U. pictorum* was observed within a range of 5–10 zebra mussels.

Oviposition was observed in ten replicates of the behavioural experiment and no oviposition was observed in a further 11 replicates. This proportion of successful ovipositions is typical for our experimental protocol (e.g. Reichard et al., 2010). Seven ovipositions occurred into unfouled *U. pictorum*. Three females attempted oviposition into a unionid fouled by five zebra mussels but two of these were not successful and eggs were laid outside the *U. pictorum* exhalant siphon, amid a zebra mussel colony. The difference in preference in terms of oviposition decision between fouled and unfouled hosts was not significant (exact binomial test, $n = 10$, $P = 0.172$), though it was significant in terms of successful ovipositions (exact binomial test, $n = 8$, $P = 0.035$). There was no significant difference in bitterling pre-oviposition behaviour expressed towards fouled and non-fouled *U. pictorum* ($n = 21$, all $P > 0.5$, Table 1).

Table 1 Results of non-parametric Wilcoxon paired tests comparing particular behaviours associated with host preference related to fouled and unfouled hosts ($n = 21$)

Behavior	<i>T</i> value	<i>z</i> value	<i>P</i>
Male inspection	29.5	0.31	0.756
Male leading	76.0	0.41	0.679
Sperm release	80.0	0.24	0.811
Female inspection	94.0	0.04	0.968
Female skimming	14.0	0.56	0.575

Discussion

We experimentally demonstrated that zebra mussels negatively affect bitterling egg load in their unionid hosts. This result corroborates previous findings from field observations and from semi-natural experiments in Britain, where both zebra mussels and the bitterling are non-native (zu Ermgassen & Aldridge, 2010). The first part of Experiment 1 used a wide range of zebra mussel infestation (0–43 individuals) and revealed a strong negative association between intensity of zebra mussel fouling and number of bitterling eggs inside a mussel host. The experiment further demonstrated that the bitterling is successfully able to oviposit into a unionid host with up to 16 zebra mussels attached to its shell near the siphons. While the second phase of Experiment 1 used a narrower range of infestation (0–9 individuals per unionid), the negative effect of infestation intensity on bitterling egg load was still significant. Note, however, that these intensities depend on zebra mussel shell size and that our estimates are valid for zebra mussels with 15–30 mm shells. Interestingly, we further detected a positive effect of *U. pictorum* shell size on bitterling egg load. While effect of shell size was always a non-significant predictor of bitterling egg and embryo load in previous studies on bitterling host choice (Smith et al., 2001, 2004) with no zebra mussels fouling, zu Ermgassen & Aldridge (2010) reported the same positive trend for the British population. We hypothesise that this effect is related to the relative proportion of zebra mussels to space on a unionid host available for zebra mussel attachment, particularly that part of the shell where the siphons are located. In larger unionids, an equal abundance of zebra mussels covers a smaller area, making it more attractive/suitable for bitterling oviposition. Another possibility is that the physiological cost is relatively lower for larger unionids (Sousa et al., 2011) and bitterling are able to detect the level of unionid stress prior to the decision to oviposit.

Importantly, we did not observe any statistically significant difference in bitterling oviposition rate or pre-oviposition behaviour between unfouled unionids and unionids fouled with five zebra mussels, despite the fact that a decrease in bitterling egg load in unionids could be demonstrated at this level of fouling (Fig. 1). Further, zu Ermgassen & Aldridge (2010) demonstrated that the same level of fouling (with a mean (\pm SE) number of zebra mussels of $4.8 (\pm 1.3)$) in

a British population of *U. pictorum* did not affect unionid glycogen reserves. This implies that the fouled and unfouled unionids in our experiment had comparable physiological status and that the main difference was likely to be a physical barrier to bitterling oviposition, represented by proximity of zebra mussels to unionid siphons. The presence of a low zebra mussel density on unionid shells, therefore, has no major effect on bitterling perception of host quality or on the decision to oviposit. We speculate that a higher intensity of zebra mussel fouling would have a negative effect on bitterling oviposition decisions as water flow from the exhalant siphon, an important cue for a bitterling decision to oviposit (Mills & Reynolds, 2003), is likely to be physically affected. Further, bitterling may be able to detect a decrease in the physiological state of the unionid host and modulate their choice accordingly (Smith et al., 2004). Bitterling oviposition decisions and their success at higher rates of zebra mussel fouling, however, have yet to be addressed and will be the subject of future studies.

The conclusion that a low level (five individuals) of zebra mussel fouling does not significantly affect bitterling oviposition rate, coupled with the fact that fewer bitterling eggs were found in unionids with such levels of infection when compared to unfouled hosts, suggests higher mortality of bitterling eggs following oviposition. This could be caused either by ejection of the eggs (Mills & Reynolds, 2002; Reichard et al., 2007a), inaccurate oviposition (with eggs laid outside the unionid exhalant siphon) (Reichard et al., 2010), or mortality related to suffocation within the unionid gills (Smith et al., 2004). It is notable that in Experiment 2 only a single oviposition into a fouled unionid was successful, compared to two inaccurate ovipositions where the eggs failed to reach the unionid gills as the ovipositor was deflected from its optimal track. All seven ovipositions into an unfouled unionid were successful. Failure to oviposit properly may have been caused by either female confusion as a result of multiple siphon streams (produced by zebra mussels) or physical interference (zebra mussels blocking the ingress of ovipositor). All inaccurate ovipositions result in egg mortality as the eggs cannot be fertilised (Smith et al., 2004) and are eaten by the spawning pair immediately following oviposition; both male and female spending several seconds after oviposition in close proximity to the host siphon (Smith & Reichard, 2005). zu Ermgassen & Aldridge (2010) hypothesised

that fouled unionids were either more likely to eject bitterling embryos or that zebra mussels affected bitterling host choice. Our data point towards a third possibility, i.e. host choice is not affected (at least at the levels of fouling in our behavioural test) but oviposition is often unsuccessful. We note that our inference is made on a small number of ovipositions and further research is needed to substantiate our suggestion.

Egg mortality related to oviposition into fouled hosts has potential population consequences as this clearly decreases the overall number of viable offspring. This effect may be largely mitigated, however, as (1) females lay many clutches, each consisting of 1–6 (typically 3) eggs, thereby bet-hedging their oviposition decisions, (2) embryo survival inside the unionid gills is density dependent (Smith et al., 2000a), with a reduction in embryo density resulting in better survival of the remaining embryos, and (3) mortality and developmental rate of juvenile bitterling is also strongly density dependent (Smith et al., 2006; Konečná et al., 2010). Negative effects of inaccurate oviposition, therefore, are only likely to be expressed when their rate is high, i.e. when zebra mussel infestation on unionids is very high. It is important to note, however, that whereas direct effects of zebra mussel presence may occur when intensity of infestation on unionids is high (corresponding to >10 zebra mussels per unionid for our study population, see Fig. 1a), indirect effects are likely to be precipitated by a decrease in unionid abundance (Mackie, 1991; Strayer & Smith, 1996) or changes in nutrient cycling (Gergs et al., 2009). Population consequences affecting bitterling and their unionid hosts caused by zebra mussel fouling requires further research. Such consequences will likely depend on prevalence, intensity of infestation, and inter-annual variability in zebra mussel fouling. A non-quantified observation from several field sites in our study area suggests large fluctuations in zebra mussel abundance over just a few years (M. Reichard, personal observation), a situation typical for several other invasive species (Simberloff & Gibbons, 2004).

Whereas our study populations of bitterling and unionids are outside the natural range of zebra mussels (Son, 2007), zebra mussel occurrence may not be historically unique in this part of the Danube basin (Orlova, 2002). Furthermore, it appears that bitterling populations have only expanded their range relatively

recently (during the Holocene) from their source population in the lower Danube (where zebra mussels are native). We have observed naturally high zebra mussel prevalence and infestation intensity on native unionids in Western Anatolia (Lake Sapanca, Turkey), where all three species are native, with remarkable inter-annual variability in fouling rate (Reichard et al., 2010; Reichard, personal observation). Bitterling from Lake Sapanca were clearly able to cope with zebra mussel fouling and eggs were regularly found in unionids (*U. pictorum*, *Anodonta anatina*, *A. cygnea*) fouled by over 50 relatively small zebra mussels (M. Reichard, unpublished data). Mortality related to inaccurate oviposition should result in very strong selection for avoidance of fouled unionids or for optimal use of the fouled hosts. In this context, it would be interesting to compare responses of several bitterling populations with a differing history of association with zebra mussels. In addition, there is a possibility that learning may decrease the rate at which fouled unionids are used, especially given that bitterling eggs are divided into many separate clutches during multiple spawnings and over a long reproductive season (Reichard et al., 2008).

Other non-native species may also affect the coevolved relationship between unionid mussels and the bitterling in Europe. *Anodonta woodiana* (Lea, 1834), a world-wide invasive Asian unionid, has recently become established in the Czech Republic and uses all available fish species as hosts for its parasitic larval stage (Douda et al., 2012). However, it avoids oviposition by the bitterling, effectively reversing the host–parasite relationship between bitterlings and unionids (Reichard et al., 2007b, 2012). A further non-native species, the North American muskrat (*Ondatra zibethicus* (L., 1766)), became established throughout Europe during the last century (DAISIE, 2011) and occurs frequently in our study area. It commonly preys on unionids (Hanson et al., 1993) but has also been reported to selectively prey on zebra mussels, discarding the live unionids to which the zebra mussels had attached themselves (Sietmann et al., 2003). Such intricate relationships between native and non-native species demonstrate that most alterations of biological communities by non-native species are complex and unpredictable, ranging from mutual facilitation (Simberloff & Van Holle, 1999) to competition between alien species.

Conclusion

We demonstrate that zebra mussel fouling is negatively associated with bitterling egg load in their unionid hosts and that, at least for lower intensities of zebra mussel infestation, the decreased bitterling egg load is most likely due to inaccurate oviposition rather than avoidance of fouled unionid hosts. In parts of the unionids range where bitterling are non-native (western Europe, including Great Britain), unionid populations must cope with two non-native parasites (bitterling and zebra mussel). In this context, zebra mussel fouling can protect its unionid host from bitterling parasitism, though the negative consequences of zebra mussel parasitism are likely to be higher than the costs related to bitterling parasitism (Reichard et al., 2006; Sousa et al., 2011). We show that indirect effects of non-native species, such as their impact on established and coevolved relationships between native species, may play an important role in their influence on native communities.

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