THE BITTERLING–MUSSEL COEVOLUTIONARY RELATIONSHIP IN AREAS OF RECENT AND ANCIENT SYMPATRY

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Host–parasite relationships are often characterized by the rapid evolution of parasite adaptations to exploit their host, and counteradaptations in the host to avoid the costs imposed by parasitism. Hence, the current coevolutionary state between a parasite and its hosts is predicted to vary according to the history of sympatry and local abundance of interacting species. We compared a unique reciprocal coevolutionary relationship of a fish, the European bitterling (*Rhodeus amarus*) and freshwater mussels (Unionidae) between areas of recent (Central Europe) and ancient (Turkey) sympatry. Bitterling parasitize freshwater mussels by laying their eggs in the gills of mussel and, in turn, mussel larvae (glochidia) parasitize the fish. We found that all bitterling from both regions avoided one mussel species. Preferences among other mussel species tended to be related to local mussel abundance rather than duration of sympatry. Individual fish were not consistent in their oviposition choices, precluding the evolution of host-specific lineages. Mussels were demonstrated to have evolved strong defenses to bitterling parasitism in the area of ancient sympatry, but have no such defenses in the large areas of Europe where bitterling are currently invasive. Bitterling avoided glochidia infection irrespective of the duration of sympatry.

KEY WORDS: Coevolutionary arm races, evolutionary lag, gentes, host race, specialization, symbiosis.

Host–parasite relationships are often characterized by the reciprocal coevolutionary "arms race" in which parasites show adaptations to maximize exploitation of their host and hosts evolve counteradaptations to avoid the costs imposed by parasitism, giving rise to a complex system of adaptations and counteradaptations (Dawkins and Krebs 1979; Thompson 1994; Poulin 2000). Host and parasite populations are often spatially structured and their relationship may evolve to different states across the range of host–parasite sympatry, depending on the historical and ecological contexts of the interaction (Gandon and Michalakis 2002; Thompson and Cunningham 2002; Benkman et al. 2003).

In parasites utilizing several hosts, each host species may require a distinct adaptation to facilitate exploitation and each may evolve distinct counteradaptations. Consequently, it may be adaptive for an individual parasite that successfully completes development in a specific host to produce progeny that tend to exploit the same host species, which may lead to the evolution of host-specific lineages (Gibbs et al. 2000; Sorenson et al. 2003; Malausa et al. 2005). However, host specialization is not an inevitable outcome of host–parasite coevolution, because it may carry costs associated with locating appropriate specific hosts. Hence it may also be more adaptive for parasites to remain as

generalists and opportunistically exploit several host species (Poulin 2000; Silva-Brandão and Solferini 2007).

Recent species expansions offer unique scenarios in which host–parasite coevolution can be compared between situations of ancient and recent sympatry between parasite and host (Lahti 2006). For example, the brown-headed cowbird (*Molothurus ater*), a brood parasite of other birds, experienced a dramatic range expansion and population increase during the last few centuries. This expansion brought it into contact with many new bird species and populations previously unexposed to brood parasitism by cowbirds. It appears that many recent host populations fail to respond to parasitism and are exploited as naive hosts that have not yet evolved adaptive responses to brood parasitism (evolutionary lag) (Rothstein 1990; Soler and Møller 1990).

Here, we use a unique study system of reciprocal parasitism in the European bitterling (*Rhodeus amarus*), a freshwater fish species that is an obligate parasite of unionid mussels, laying its eggs in the gill cavity of the mussel. The mussels used for oviposition have larval stages (termed glochidia) that are obligate parasites of freshwater fish. Both partners display distinctive morphological, physiological, and behavioral adaptations for host exploitation and counteradaptations against being parasitized by the other partner (Smith et al. 2004). The occurrence of bitterling in Central and West Europe is recent (Kozhara et al. 2007; Van Damme et al. 2007) and appears to have coincided with an increase in global temperature at the end of the Little Ice Age (cf. 1850 AD) (Van Damme et al. 2007) when bitterling expanded from the Pontic region (Bohlen et al. 2006). No such range shift was recorded in unionid mussels, which have been a stable component of the freshwater fauna of Central and West Europe throughout the Holocene (Kennard and Woodward 1903; Kennard 1924; Watters 2000). Regardless of the absolute timing of the bitterling expansion to Central and West Europe, the mussel populations used by the bitterling for oviposition in Central Europe are hosts with a recent sympatry in contrast to bitterling populations from the Pontic region, where bitterling and mussels have evolved in sympatry for at least 2 million years (Bohlen et al. 2006; Kozhara et al. 2007; Reichard et al. 2007b; Van Damme et al. 2007).

During reproduction male bitterling court females and lead them to mussels for oviposition. Females inspect mussels and, if they choose to oviposit, insert a long ovipositor into the mussel exhalant siphon to place their eggs deep inside the mussel gill cavity. Males fertilize the eggs by releasing sperm into the inhalant siphon of the mussel, so that water filtered by the mussel carries the sperm to the eggs. Preoviposition sperm releases, whereby males ejaculate into the siphon of a mussel before a female spawns, are a common feature of male courtship. One to six large elliptical yolk-rich eggs are laid during each spawning. The eggs hatch in 36 h and embryos develop inside the mussel for three to six weeks before departing as actively swimming juve-

niles (Smith et al. 2004). Mussels often contain eggs and embryos from multiple ovipositions, with up to 250 bitterling embryos in a single mussel (Smith et al. 2004). Bitterling embryos can inflict significant fitness costs on mussels (Reichard et al. 2006) through damage to gill epithelium, competition with mussels for oxygen, and disruption of water circulation over the gills (Stadnichenko and Stadnichenko 1980; Smith et al. 2001; Mills and Reynolds 2002).

Unionid mussels occupy benthic freshwater habitats and filter water through their gills to obtain food and oxygen. Water enters the mussel gill through an inhalant siphon leading to the mantle cavity. Water is passed through the gills to their inner surface and is channeled via the water tubes to the suprabranchial cavity from which it is expelled through the exhalant siphon (Bauer and Wächtler 2000). The exhalant siphon forms part of the mantle tissue and is not physically attached to the suprabranchial cavity. Hence there is no physical connection between the gills and the exhalant siphon and water circulating inside the gills enters the mantle cavity before reaching the exhalant siphon. Female unionids brood their glochidial larvae in modified sections of the gill, termed marsupia, from which they discharge mature glochidia into the water column. Glochidia are composed of a tiny (0.10 mm in *Unio* spp.) hinged valve that snaps shut on contact with fish tissue, typically attaching to their gills or fins (Blažek and Gelnar 2006). When attached, the glochidia must be encysted by host tissue to complete their development and remain attached for several weeks (Bauer and Wächtler 2000). High levels of infection by glochidia can be lethal to a fish (Meyers and Millemann 1977).

Four species of unionid mussels commonly co-occur with bitterling in Central Europe and all of them may be used for oviposition; *Unio pictorum*, *Unio tumidus*, *Anodonta anatina*, and *Anodonta cygnea*. *Unio crassus* co-occurs with bitterling in the Pontic region where it replaces *U. tumidus*. Host mussel species differ in the anatomy of their gills, oxygen consumption, and flow rate of water circulating through their gills (Smith et al. 2001; Mills et al. 2005). For example, water tubes (the interlamellar spaces in the mussel gill where bitterling eggs reside) of *Anodonta* spp. are more complex than those of *Unio* spp. (Liu et al. 2006). *Anodonta cygnea* has significantly wider gill septa and consumes more oxygen than the other three species (Smith et al. 2001; Mills et al. 2005). These and other differences among mussels may be important for the survival of bitterling eggs and embryos, because the most important source of their mortality is egg ejection from the mussel gills and suffocation (Mills and Reynolds 2002; Kitamura 2005).

Host mussels have evolved counteradaptations that enable them to eject developing bitterling eggs and embryos (Smith et al. 2004); mussels rapidly contract their valves and expel a stream of water that can dislodge bitterling eggs and embryos from their gills. Bitterling make sophisticated oviposition

decisions that limit ejections (Smith et al. 2000; Mills and Reynolds 2002). Ejections typically occur within the first six days of embryo development (Nagata 1985; Mills and Reynolds 2002; Kitamura 2005). Immediate ejections (within a few seconds of oviposition) are also an important source of egg mortality (Reichard et al. 2007a) and strongly correlate with overall ejection rate (Reichard et al. 2007b). Ejection of bitterling eggs by mussels is most frequent at the centre of bitterling diversity in Asia (Reichard et al. 2007a), where more than 40 species of bitterling are recognized and where the bitterling-mussel association is ancient, with bitterling fossils dated to 16 millions years ago (Tomoda et al. 1977).

In central Europe, bitterling almost entirely avoid glochidial infection (Reichard et al. 2006), though diverse outcomes are reported from other parts of the bitterling-mussel sympatry. Although adult and embryo bitterling (*Rhodeus* spp.) are often heavily infected by glochidia in the Caucasian region and the Russian Far East (N. Bogutskaya, pers. comm.), *Rhodeus ocellatus* from Southern and Eastern China appear to partially avoid glochidia of *Anodonta woodiana* (Dudgeon and Morton 1984; Fukuhara et al. 1986; Reichard et al. 2006).

In this study, we tested the general hypothesis that the European bitterling from a region of ancient sympatry with their mussel hosts (the Pontic region of Turkey) are under stronger selection to avoid mussel counteradaptation compared to bitterling populations from regions of more recent sympatry where they encounter naive hosts (Central Europe) (Soler and Møller 1990). Consequently, we predicted that bitterling from the region of ancient sympatry would have evolved a stronger preference for particular mussel species, either at the population level or at the level of individual fish (expressed as individual consistency of mussel species choice). In the region of ancient sympatry, we further predicted stronger individual host preferences (i.e., consistency) in bitterling from sites with high mussel abundance and diversity, and population and individual preference for sympatric mussel species in bitterling from sites that contained only a single mussel species. For mussel responses to bitterling oviposition, we predicted that mussels would have stronger defenses in the region of ancient sympatry, either as a direct response to prevent oviposition or through responses that would ameliorate the cost of hosting eggs. For infection of bitterling by mussel glochidia, however, we predicted no difference in the rate of infection between regions.

This prediction derives from the unequal costs of parasitism for bitterling and mussels. Bitterling are not the only potential host of glochidia, indeed they are often a minor component of the fish assemblages in which they occur and rarely represent a significant pool of potential hosts. In contrast, bitterling only occur in sympatry with mussels, and exposure to glochidia represents a potentially substantial cost.

Methods

STUDY SITES AND POPULATIONS

Data were collected for populations from the River Ballica (41°00′N, 29°25′E) and Lake Sapanca (40°42′N, 30°15′E) in Turkey, and River Kyjovka (48°47′N, 17°01′E) and Lake Bazina (48°38′N, 16°56′E) in the Czech Republic. Populations were selected based on habitat characteristics and the abundance of unionid mussels (Table 1), with a low and high mussel density site from each region. Bitterling density was high at all sites, with a range of bitterling to mussel ratios typical of those seen in previous population surveys (Smith et al. 2000). To estimate egg densities within mussels, and thereby the degree of competition for spawning sites (egg crowding), 10 *A. anatina* and 10 *U. pictorum* mussels were exposed to bitterling spawning at each study site over the period of peak bitterling spawning for 3–4 h and expressed as the number of eggs laid in a mussel per 1 h of exposure (Table 1).

HOST PREFERENCE AND CONSISTENCY

Host preference and consistency of preferences were tested in experimental aquaria (50 × 30 × 30 cm). Each morning, four haphazardly chosen females in spawning condition were selected from a stock of recently collected fish (held in 200 L tanks) and placed individually in a transparent plastic cup floating in the experimental aquarium in which a single haphazardly captured male from the same population was housed. Each experimental aquarium contained four mussels in separate sand-filled plastic cups aligned in a row in a randomly selected position. In Turkey, the mussel species presented were *A. anatina* (mean ± SD mussel size = 94 ± 15 mm), *A. cygnea* (119 ± 17), *U. crassus* (65 ± 6) and *U. pictorum* (73 ± 7). In the Czech Republic, *A. anatina* (81 ± 12 mm), *A. cygnea* (118 ± 20), and *U. pictorum* (77 ± 9) were used along with *U. tumidus* (73 ± 13), which replaces *U. crassus* in Central and West Europe. Once the male started to

Table 1. Characteristics of four study populations and their collection sites. Mussel species present at sites are ranked from highest to lowest abundance; rarely collected species are in parentheses.

Site	*L. Sapanca*	*R. Ballica*	*L. Bazina*	*R. Kyjovka*
Sympatry	Ancient	Ancient	Recent	Recent
Mussel species	AA, UP, (AC)	AA	AA, (AC)	AA, UP, UT
Egg crowding (mean±SE)	Low (0.18±0.10)	High (4.69±0.80)	High (1.02±0.38)	Low (0.31±0.11)

court the female she was gently released from the cup and observations started. We collected data on fish behavior toward each mussel until oviposition (or for a maximum of 40 min, if oviposition did not occur) using established protocols (Smith et al. 2001; Reichard et al. 2004). We collected data on male inspection of the mussel siphon, male leading of the female to a specific mussel, sperm release into the mussel inhalant siphon, female inspection of the mussel siphon, and female skimming (deceptive oviposition without releasing eggs). These behaviors represent male and female investment in specific mussels (Reichard et al. 2007a). If oviposition occurred, a mussel that received eggs was observed for another 15 sec to record any immediate ejections of eggs (Reichard et al. 2007a, b). Four replicates with four pairs (each in a separate tank) were completed in each round of testing. After completion of an observation, mussels were removed, measured for shell length and checked for the presence of glochidia and bitterling eggs using a mussel-opening device (Kitamura 2005). A new set of mussels, arranged in a predetermined random order, was placed in the tank. Females were captured, transferred into another tank in a plastic cup, and tested again with a new male and new set of mussels. A minimum time between two tests with the same female was 20 min to ensure that females were always ready to oviposit (Smith et al. 2004). A total of 16 replicates with four males and four females were completed, with four replicates completed for each of the test fish (always with a different partner). A total of 16 males and 16 females (64 observations) were tested from each bitterling population over the period of spawning (mid April to mid May). Additional oviposition choice tests were conducted for the Lake Sapanca population, which showed a low oviposition rate during the initial round of the experiment. A different set of fish and mussels were used, though the tank setting was identical.

MUSSEL RESPONSES

During exposure of mussels to natural spawnings to estimate egg densities in mussels among sites (Table 1), the number and proportion of bitterling eggs deposited in mussel mantle cavities (instead of their normal positioning in the water tubes or suprabranchial cavity, which is essential for completion of embryo development) was recorded. The aperture length (distance between posterior end of the exhalant siphon and the anterior end of suprabranchial cavity) was measured to the nearest 0.01 mm nondestructively using a mussel-opening device and electronic calipers. The aperture is a gap between the gill and mantle tissue through which the bitterling ovipositor passes during oviposition. If the ovipositor enters the aperture, the eggs are laid inside the mantle cavity instead of into the gills. Hence a longer aperture is predicted to result in more misplaced ovipositions. A total of 25 mussels each of *A. anatina* and *U. pictorum* were measured from both regions. In addition, the response of mussels to a tactile

stimulus was measured as the time between exhalant siphon stimulation and siphon closure. Mussels were placed in sand-filled pots and allowed to settle in a large aquarium with a 200-mm water depth. A metal rod with a blunt tip (diameter 3 mm) was used to mimic the contact of a bitterling ovipositor during spawning. The response time (to the nearest 0.1 sec) was estimated using footage from a digital video camera (Olympus μ Tough Olympus, Tokyo, Japan) replayed, frame-by-frame, using Quick Time Player. Data on aperture length and tactile reaction could only have been measured for mussels from high mussel density sites within each region; at low mussel density sites too few mussels were collected to obtain adequate sample sizes.

GLOCHIDIA INFECTION

Experimental exposure to glochidia was conducted in 40 L aquaria, each containing locally collected *U. pictorum*. The period of glochidia release by *U. pictorum*, unlike that of *A. anatina*, broadly overlaps with the bitterling reproductive season when the fish are most susceptible to infection as a result of their mussel-oriented behavior. Each aquarium was stocked with two individual bitterling, a control local cyprinid from another subfamily (Leuciscinae), and tubenose goby (*Proteorhinus marmoratus*), a species known to be highly susceptible to glochidia infection (Koubková and Baruš 2000). Different control cyprinid species were selected to represent a locally abundant cyprinid, the Dnieper chub (*Petroleuciscus borysthenicus*) in Turkey and rudd (*Scardinius erythrophthalmus*) in the Czech Republic. Four *U. pictorum* with mature glochidia in their gills were added to each of 10 experimental aquaria and left to discharge glochidia. After four days, mussels were removed and 24 h later one individual of each fish species was removed, killed with an overdose of anesthetic and fixed in a 6% formaldehyde solution. The remaining fish were removed and fixed after a further three days. Following physical attachment of glochidia to a fish host, encystation by host tissue occurs within three days or the fish may eliminate the parasite (Wächtler et al. 2000). Hence, our second collection of fish represented encysted glochidia. An additional seven fish per species and region were fixed immediately after fish collection to provide an estimate of natural infection rates prior to experimental exposure. Only four control *P. marmoratus* were examined in the Czech Republic due to our failure to catch an adequate number. Infection rates prior to experimental exposure were negligible (a total of one glochidium in Turkey, three glochidia in the Czech Republic). Glochidia were counted on the fish body, fins, gills, and gill cover under a dissecting microscope.

Statistical analyses were conducted using R (R Core Development Team 2007). Generalized linear models were used to test oviposition preference (binomial error structure), behavioral preference (quasi-Poisson error structure), and glochidia load (quasi-Poisson error structure). Differences in glochidia load among fish

species were tested for each region separately, different control cyprinid species having been used in each. In mussel preference tests, only the first behavior record for each fish was used, to avoid pseudoreplication (the sum of behaviors across all four observations gave concordant results). General linear models were used to test differences in mussel defense mechanisms and consistency in behavioral preference tests. To analyze behavioral consistency, Kendall concordance coefficients (*W*) were calculated for each individual fish (for each behavior separately) and subjected to analysis of variance (ANOVA) after arcsine transformation. Binomial tests and contingency tables were used to test oviposition preference and its consistency. For preferences, random host use served as the null hypothesis (probability of host use 0.25 for each species). Based on mussel preference results, consistency was subsequently tested using three host species (*A. cygnea* was avoided by bitterling in both regions). Hence, for statistically consistent oviposition choice, the probability of using the same host species in sequential choices should have been significantly higher than 0.33, whereas strict consistency would yield a probability of the same host use equal to 1. Egg ejection rate was compared between regions using binomial tests.

Results

HOST PREFERENCE

Bitterling significantly avoided *A. cygnea* for oviposition, but used all other host species readily (mussel effect: deviance = 78.95, df = 3, *P* < 0.001; Fig. 1). There was no effect of mussel size (deviance = 2.30, df = 1, *P* = 0.13) or presence of glochidia (deviance = 0.80, df = 1, *P* = 0.37) on bitterling oviposition choice, but an interaction between mussel species and population showed that preference for particular mussel species differed among bitterling populations (deviance = 17.28, df = 9, *P* = 0.04). After exclusion of *A. cygnea* from the dataset, preferences of bitterling from sites with high mussel densities were stronger (Kyjovka: deviance = 7.18, df = 2, *P* = 0.028; Sapanca: deviance = 5.73, df = 2, *P* = 0.057) than from low mussel density sites (Bazina: deviance = 2.30, df = 2, *P* = 0.318; Ballica: deviance = 2.94, df = 2, *P* = 0.230). Kyjovka bitterling avoided oviposition in *A. anatina*, whereas Sapanca bitterling rarely chose *U. crassus* (Fig. 1).

Behavioral preferences were generally congruent with oviposition choice. Details of statistical tests (GLLM with quasi-Poisson distribution) are summarized in Table 2A. Overall, fish paid significantly less attention to *A. cygnea*. Significant interactions between site and mussel species revealed that fish from the Kyjovka inspected *U. tumidus* most often compared to a high inspection rate of *A. anatina* by females from populations in Ballica and Bazina (Table 2). Although fish from Sapanca inspected mussels readily and males led females to them, significantly fewer
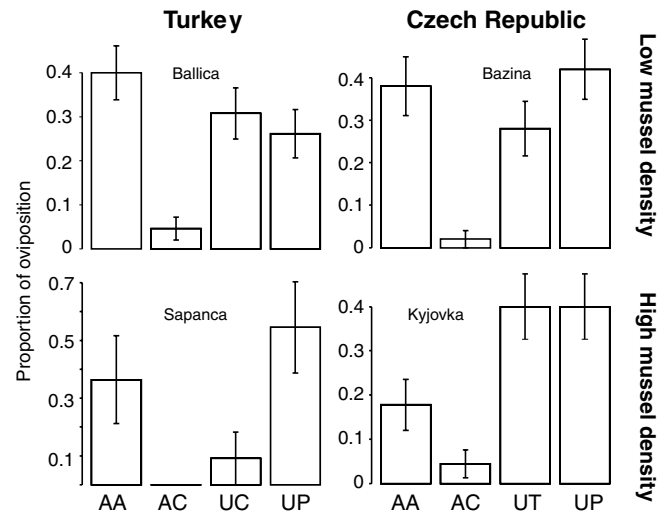
Figure 1. Host preference of bitterling from four study populations in terms of the proportion of eggs laid in each of study mussel species (AA, *A. anatina*; AC, *A. cygnea*; UC, *U. crassus*; UP, *U. pictorum*; UT, *U. tumidus*). Preferences of all populations are significant if all four species are considered in the analysis. However, only the Kyjovka population showed a significant preference for *U. pictorum* and *U. tumidus* over *A. anatina* if only three mussel species are considered (details in text).

sperm releases and skimming behavior were recorded for this population.

INDIVIDUAL CONSISTENCY IN HOST PREFERENCE

There was no consistency in the mussel preferences of either males or females from any bitterling population (Table 3). There was also no difference among populations in the consistency of behavioral preferences (Table 2B). The strongest behavioral consistency was recorded for male inspection of mussel siphons (Friedman test, $\chi^2 = 49.38$, df = 3, $P < 0.001$); other measures of behavioral preference were not consistent (Fig. 2).

HOST RESPONSES

In *U. pictorum*, the rate of immediate egg ejection was 2.5 times higher at sites of ancient than recent sympatry (44.4% compared to 17.8%), and this difference was significant (binomial test, $\chi^2 = 4.73$, df = 1, $P = 0.030$), although no difference was observed in *A. anatina* (binomial test, $\chi^2 = 0.001$, df = 1, $P = 0.976$; 21.8% overall).

Significantly more eggs were found in the mantle cavity, outside the mussel gill, at sites of ancient sympatry in both *A. anatina* (binomial test, $\chi^2 = 129.6$, d.f. = 1, $P < 0.001$) and *U. pictorum* (binomial test, $\chi^2 = 160.3$, d.f. = 1, $P < 0.001$). This phenomenon was observed in 63% of *A. anatina* and 53% of *U. pictorum*, affecting 34% of recently laid eggs in *A. anatina* and 22% of recently

Table 2. Results of (A) the effect of population identity, mussel species, and their interaction on behavioral preference measures in four bitterling populations using GLLM analysis with quasi-Poisson distribution. Presence of glochidia in mussel gills, mussel shell size, and presence of bitterling eggs were included in the analysis as uncontrolled sources of variation. (B) The effect of the length of the bitterling-mussel association and mussel density on behavioral consistency (ANOVA on Kendall coefficients of concordance). Smaller degrees of freedom for leading and skimming behavior come from a failure of some fish to perform these behaviors.

	(A) Behavioral preference				(B) Behavioral consistency		
	df	F	P		df	F	P
Leading							
Locality	3,235	2.34	0.074	Sympatry	1,59	1.24	0.271
Mussel species	3,235	7.90	<0.001	Density	1,59	0.16	0.688
Glochidia	1,235	0.01	0.924	Symp*dens	1,59	1.64	0.205
Mussel size	1,235	1.36	0.245				
Egg presence	1,235	1.07	0.301				
Locality × mussel sp	9,235	0.90	0.530				
Sperm release							
Locality	3,235	3.03	0.030	Sympatry	1,60	1.20	0.277
Mussel species	3,235	10.39	0.001	Density	1,60	0.16	0.686
Glochidia	1,235	0.29	0.589	Symp*dens	1,60	0.91	0.344
Mussel size	1,235	0.01	0.912				
Egg presence	1,235	0.22	0.635				
Locality × mussel sp	9,235	1.55	0.132				
Male inspection							
Locality	3,235	4.47	0.005	Sympatry	1,60	0.14	0.706
Mussel species	3,235	27.36	<0.001	Density	1,60	1.34	0.251
Glochidia	1,235	1.08	0.299	Symp*dens	1,60	0.33	0.570
Mussel size	1,235	1.06	0.305				
Egg presence	1,235	0.002	0.964				
Locality × mussel sp	9,235	2.84	0.003				
Female inspection							
Locality	3,235	2.35	0.073	Sympatry	1,59	0.25	0.619
Mussel species	3,235	6.33	<0.001	Density	1,59	1.93	0.170
Glochidia	1,235	1.64	0.201	Symp*dens	1,59	1.57	0.215
Mussel size	1,235	0.05	0.826				
Egg presence	1,235	0.19	0.667				
Locality × mussel sp	9,235	2.70	0.005				
Skimming							
Locality	3,235	6.55	<0.001	Sympatry	1,48	0.07	0.790
Mussel species	3,235	3.27	0.022	Density	1,48	0.83	0.367
Glochidia	1,235	0.07	0.792	Symp*dens	1,48	0.25	0.618
Mussel size	1,235	0.79	0.374				
Egg presence	1,235	5.76	0.017				
Locality × mussel sp	9,235	1.51	0.146				

laid eggs in *U. pictorum* (Fig. 3). In four *A. anatina*, all the eggs were positioned in the mantle cavity. In the area of recent sympatry, eggs were rarely positioned in the mantle cavity; only two eggs in a single *A. anatina* and a single egg in *U. pictorum*.

Mussels from sites of ancient sympatry responded to a tactile stimulus with a more rapid closure of their exhalant siphons (two-way ANOVA, $F_{1,42} = 18.62, P < 0.001$), although there was no difference between *A. anatina* and *U. pictorum* ($F_{1,42} = 2.96, P =$

0.093), and no interaction between species and region ($F_{1,42} = 0.21, P = 0.650$).

Mussels at sites of ancient sympatry had longer apertures (two-way analysis of covariance [ANCOVA], with mussel size as a covariate, $F_{1,95} = 23.02, P < 0.001$) and *U. pictorum* had longer apertures than *A. anatina* ($F_{1,95} = 36.65, P < 0.001$). There was no significant interaction between these variables ($F_{1,95} = 0.64, P = 0.427$).

Table 3. Consistency of bitterling oviposition behavior measured as repeated use of the same mussel species in two consecutive ovipositions with different individual mussels (cons). Given that *A. cygnea* was avoided in all populations, consistency was defined as the probability of using the same host species at a significantly greater probability than 0.33.

	Females					Males				
	Cons	Total	Prop	χ^2	P	Cons	Total	Prop	χ^2	P
Ballica	13	40	0.33	0.008	0.927	15	40	0.38	0.208	0.648
Sapanca	1	4	0.25	–	–	–	–	–	–	–
Bazina	11	32	0.34	0.010	0.919	10	33	0.30	0.091	0.763
Kyjovka	9	29	0.31	0.046	0.830	8	28	0.29	0.190	0.663

GLOCHIDIA INFECTION

Glochidial infection of bitterling was low compared to control species in both regions (GLLM with quasi-Poisson distribution, $F_{2,57} = 9.73$, $P < 0.001$ and $F_{2,56} = 17.01$, $P < 0.001$ for sites of ancient and recent sympatry, respectively). There was no effect of host size on the number of glochidia on a fish ($F_{2,55} = 1.91$, $P = 0.173$ and $F_{1,54} = 0.27$, $P = 0.608$; ancient and recent sympatry, respectively). There was also no significant difference between the glochidia counts 24 or 72 h after infection from the site of ancient sympatry ($F_{1,56} < 0.01$, $P = 0.979$), although there was a significant decrease in the site of recent sympatry ($F_{1,56} = 5.29$, $P = 0.026$; Fig. 4).

The prevalence of glochidia was similarly low in bitterling compared to control species and not significantly different between sites of ancient and recent sympatry, although no decrease in prevalence was observed between sampling periods. Prevalence in the region of ancient sympatry was 90% in *P. marmoratus*, 75% in the control cyprinid, and 45% in bitterling, and in recent sympatry 84% in *P. marmoratus*, 80% in control cyprinid, and 25% in bitterling.

Discussion

We compared the reciprocal coevolutionary relationship between bitterling fish and freshwater mussels in areas of ancient and recent sympatry. Our key findings were: (1) all bitterling populations from both regions avoided one species of host mussel, but used the other three mussel species readily; (2) mussel defense was stronger in a region of ancient sympatry than of recent sympatry; (3) bitterling avoided glochidia infection irrespective of the duration of sympatry.

We found that all bitterling populations avoided oviposition in *A. cygnea*, which is consistent with previous studies from Western and Central Europe (Reynolds et al. 1997; Smith et al. 2000). *Anodonta cygnea* has the widest gill septa, consumes most oxygen, and has the highest speed of water circulation through its gills than the other three species (Smith et al. 2001; Mills and Reynolds 2002; Mills et al. 2005), with the result that the mortality rate of bitterling embryos in this mussel is significantly higher than the
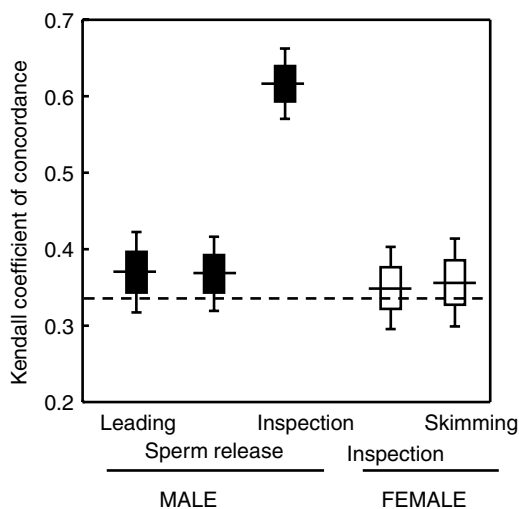
Figure 2. Individual consistency of particular behaviors directed toward specific mussels across four observations of each individual fish (with different individual mussel and a different mating partner in each observation) measured as Kendall coefficient of concordance (mean ± 1 standard error (box) and confidence interval (whiskers)). Male behavior is represented by black boxes, female behavior by white boxes.
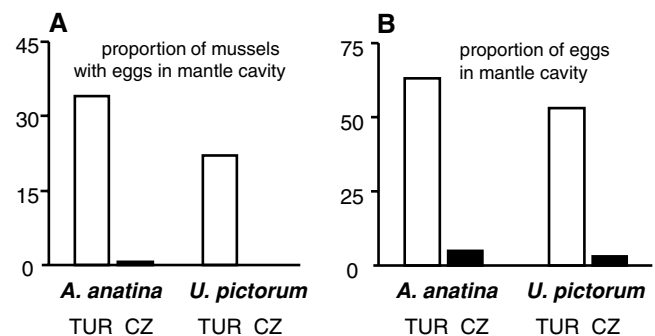
Figure 3. The proportion of (A) mussels with at least a single egg in its mantle cavity, and (B) the overall proportion of eggs in the mantle cavity (across all mussels pooled) for *A. anatina* (AA) and *U. pictorum* (UP) in areas of ancient (TUR) and recent (CZ) sympatry.
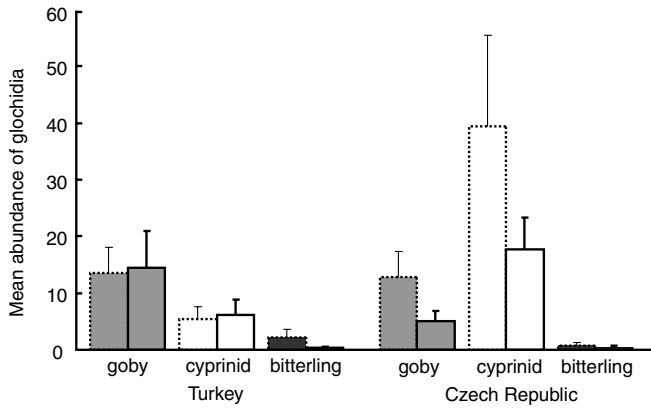
Figure 4. Glochidia load of bitterling and two control species (goby—*Proteorhinus marmoratus*, cyprinid—*Petroleuciscus borysthenicus* in Turkey and *Scardinius erythrophthalmus* in the Czech Republic) in terms of mean glochidia abundance per dissected fish one day (dashed line) and four days (solid line) after exposure to infection.

other species available (Smith et al. 2000, 2001). The finding that host species preferences are congruent across the European bitterling range suggests that there are clear physiological constraints to host quality outside the coevolutionary relationship, with bitterling embryo adaptations appearing suboptimal for development in *A. cygnea*. Despite their low quality as hosts, bitterling embryos are able to complete development in *A. cygnea* and they are used as hosts, albeit at a low frequency (Reynolds et al. 1997; Smith et al. 2000).

Bitterling preferences for the remaining three mussel species varied only slightly among populations. A prediction that bitterling from sites with only a single host species would prefer sympatric mussels was not confirmed, strengthening the conclusion that the mussel preferences of the European bitterling are not shaped by learning or imprinting. Imprinting on natal host species is reported to maintain host-specific lineages in African indigobirds (Sorenson et al. 2003), but in other study systems, proximate mechanisms of host discrimination are more difficult to test. For example, it is known that female European cuckoos (*Cuculus canorus*) consistently lay their eggs into nests of host species in which they were raised, leading to the evolution of female host-specific races (called gentes) (Gibbs et al. 2000), although the underlying mechanism is not known despite a substantial research effort (reviewed in Langmore and Kilner 2007).

Populations did not differ in their consistency of host preference, and individual males and females were inconsistent with respect to host species in their behavioral and oviposition preferences. We predicted a higher level of consistency in populations of ancient sympatry, given the number of bitterling species or subspecies that are specialized in their use of host species in Asia

(Liu et al. 2006; Reichard et al. 2007a; Kitamura et al. 2009) and frequent occurrence of host-specific lineages in avian brood parasites (Kruger et al. 2009), plant-feeding insects (Drès and Mallet 2002), and other comparable systems (e.g., Munday et al. 2004). However, this prediction was not satisfied and may simply reflect an overall shorter period of association between bitterling and mussels in Europe, compared with Asia (Tomoda et al. 1977). Alternatively, the benefits associated with consistent host preferences may be exceeded by the costs of specializing on a single or limited number of hosts.

Mussel defenses were strikingly stronger in the region of ancient sympatry. We previously suggested that bitterling in Central Europe use relatively naive hosts that have not evolved strong counteradaptations to defend against parasitization by bitterling (Reichard et al. 2007b). Based on mussel defenses against bitterling parasitism in Asia (Reichard et al. 2007a), we predicted egg ejection behavior to be the most important adaptation of mussels in Turkey. This prediction was met in *U. pictorum*, with 2.5 times more frequent ejections of eggs following oviposition in Turkish populations in comparison with Czech populations, although not in *A. anatina*. Strikingly, we observed high numbers of eggs in the mantle cavity of mussels of both species in Turkey; a situation rarely observed in European mussels. Accordingly, we tested the two most likely mechanisms that could be responsible for the presence of bitterling eggs in the mantle cavity. These experiments demonstrated that in the region of ancient sympatry, mussels closed their siphons significantly more quickly in response to tactile stimulus, which would tend to interrupt the correct insertion of the ovipositor into the mussel gill cavity. In addition, mussel internal anatomy differed between regions, with a significantly larger distance between the gill and mantle tissues (termed aperture length) in the area of ancient sympatry. We presumed that the larger aperture length more frequently leads to the misplacement of ovipositor during spawning and may further increase incidence of the egg deposition outside the gills.

We assumed that the difference in mussel defense against bitterling oviposition represented enhanced mussel resistance in the coevolutionary interaction. However, our experimental design did not reveal whether the observed resistance is specific to the observed sympatric combination or whether it is a broad resistance mechanism against bitterling from any region. To investigate the specificity of mussel response, a cross-infection experiment testing the response of mussel populations to bitterling oviposition between regions of recent and ancient sympatry must be employed. Such experiments could further unambiguously test other hypotheses, for example whether bitterling from regions of recent sympatry have evolved an enhanced ability to use mussels more effectively than in regions of ancient sympatry (although interpopulation differences in mussel behavior and anatomy are not congruent with this view). The prediction for cross-infection

experiments is that bitterling from regions of recent sympatry would face the same resistance from mussels in ancient sympatry as local bitterling if only mussel resistance differs between the regions. However, if both partners coevolve and bitterling from the region of ancient sympatry have partly compensated for enhanced mussel defense, bitterling from the region recent sympatry are expected to be even less successful in their utilization of mussels from the region of ancient sympatry.

Bitterling avoided glochidia infection in both regions. The ability to avoid glochidia infection could be either acquired or genetic (or both). Bitterling embryos are incubated in intimate contact with mussel tissue throughout their development, although rarely directly with glochidia. Fish develop immunity to glochidia infection and become less susceptible to repeated exposure (Jansen et al. 2000; Rogers and Dimock 2003). A significant proportion of experimental bitterling probably developed in *A. anatina*, but all appeared unsusceptible to *Unio* glochidia infection, suggesting that any potential acquired immunity applies across host mussel genera. Alternatively, there may be a genetic component to resistance. Bitterling that expanded from areas of long-term sympatry to exploit naive mussel populations do not appear to have experienced a relaxation in selection pressure to avoid glochidia infection. This finding suggests that the cost of harboring glochidia would be high enough to generate an evolved genetic response. Whether the response is acquired or genetic can perhaps be tested using in vitro spawning and artificial exposure of laboratory-reared bitterling. The mechanism of resistance to glochidia by bitterling is not known.

In conclusion, we established that mussels have evolved strong defenses to bitterling parasitism in an area of ancient sympatry, whereas their defenses appear to be more relaxed in an area of relatively recent sympatry. This finding contradicts a previous conclusion that mussel defense behavior might not have been shaped by coevolutionary interactions with bitterling and is rather a byproduct of mussel physiology (Mills et al. 2005). We further demonstrated that bitterling adaptation to mussels is likely to be generalized, with no host specificity at the individual or population level. Two explanations may account for this finding. First, generalized bitterling embryo adaptations (a unique wedged shape, presence of scaly tubercles on the yolk, positive rheotaxis and negative phototaxis) (Smith et al. 2004) are a general response to prevent ejections with similar effectiveness among all three preferred host species. Second, there are constraints on host specialization due to temporal changes in the abundance of particular mussel species or migration between bitterling populations with different host availability (Abrams and Kawecki 1999; Gandon and Michalakis 2002). Notably, there is a considerably greater diversity of mussel and bitterling species in Asia where several bitterling species have specialized to exploit a single mussel species (Liu et al. 2006; Reichard et al. 2007a). One possibility

is that use of mussel species by bitterling may be relatively generalist at the edge of the range of bitterling-mussel sympatry, where mussel responses are limited. In contrast, at the centre of their association, there may be stronger selection for specialization by bitterling where mussel responses are stronger and interspecific competition for mussels among bitterling species widespread.

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