A possible evolutionary lag in the relationship between freshwater mussels and European bitterling

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(Received 6 March 2006, Accepted 16 October 2006)

The coevolutionary dynamics between European bitterling Rhodeus amarus and freshwater unionid mussels, which the former parasitize by laying eggs on their gills, were tested. In a series of experiments fish preferences and mussel responses were compared in parasites and hosts of recent (Europe) and ancient (Asia) sympatry. Rhodeus amarus readily oviposited on the gills of all mussel species tested. Fish that laid their eggs on the gills of Asian Anodonta woodiana, however, suffered a dramatic reduction in reproductive success compared to fish that oviposited on the gills of European mussels: Unio pictorum, Unio tumidus, Anodonta anatina and Anodonta cygnea. This difference was the result of egg ejection behaviour by mussels rather than the unsuitability of the internal gill environment for European bitterling embryo development. The ejection response of mussels with a long sympatry with European bitterling was considerably more pronounced than that of mussels with a substantially shorter sympatry. The data support a coevolutionary arms race between bitterling and mussels and point to an evolutionary lag in the relationship between R. amarus and its European mussel hosts.

Key words: Anodonta; coevolution; host ejection; oviposition choice; Rhodeus sericeus; Unio.

INTRODUCTION

Interspecific relationships that involve complex networks of interactions are ideal models for studies of adaptation; such systems are often highly dynamic and permit rapid coevolutionary change to be observed over relatively short periods. Many of the theoretical (Rothstein, 1975; Zahavi, 1979; Takasu et al., 1993) and empirical (Davies & Brooke, 1988, 1989; Rothstein, 1990; Lotem et al., 1992; Øien et al., 1996) insights into interspecific interactions have come from
studies of host-parasite systems. The dynamics of host-parasite systems are particularly variable, while some represent rapidly coevolving ‘arms races’ between species (Dawkins & Krebs, 1979; Davies & Brooke, 1989; Payne, 1997; Rothstein & Robinson, 1998), others appear not to coevolve (Frank, 2000) and their relationships may be shaped by asymmetries in coevolution (Lachin & Guillemaud, 2005). While parasites may utilize the most suitable hosts and show preferences and adaptations for using them, adaptive responses in hosts may be lacking, especially when the costs associated with parasitism are negligible, or the probability of parasitism limited (Jokela et al., 2000; Lachin & Guillemaud, 2005). In such situations, observed parasite preferences may be linked to traits that signal host quality for parasite development (Smith et al., 2000a).

For situations when host and parasite do coevolve, two influential theoretical models have been used to understand the evolution of adaptations. Both models assume that an adaptive response by a host to a parasite is dependent on ancestral genetic variation for a response. The evolutionary lag model predicts host defences to be weak or absent if there has not been sufficient time for defences to evolve and spread in a population (Rothstein, 1975; Holland et al., 2004). The evolutionary equilibrium model addresses the costs and benefits to both the host and parasite (Zahavi, 1979; Lotem et al., 1992; Takasu et al., 1993) and predicts a dynamic relationship between host and parasite in their responses to each other, with the relative costs and benefits to the parasite and host being central to resolving how the equilibrium is balanced. In this model, host defences may have sufficient time to evolve and spread, but are limited by the costs that defence might incur. The two models, however, are not mutually exclusive and may describe host-parasite systems at different stages of a coevolutionary arms race.

Here, the relationship between the European bitterling Rhodeus amarus (Bloch) (Cyprinidae) and unionid freshwater mussels was studied. Rhodeus amarus is a freshwater fish that parasitizes mussels by laying its eggs on the gills of living mussels. Bitterling display remarkable morphological, physiological and behavioural adaptations for using mussels as spawning sites and represent a valuable model in behavioural and evolutionary ecology (Smith et al., 2004). Female bitterling possess long ovipositors that they use to place their eggs into the gill cavity of a mussel through the mussel’s exhalant siphon. Between one to six eggs are released each spawning, and R. amarus spawn repeatedly over the 2-month reproductive season; a single mussel may harbour >250 (typically 40) embryos on its gills. The embryos develop inside the mussel for c. 1 month and during that time compete with the host mussel for oxygen (Smith et al., 2001) and reduce water circulation over the mussel’s gills (Mills et al., 2005). Mussels infected with bitterling embryos suffer significantly reduced growth rates and reproductive success (Reichard et al., 2006).

Rhodeus amarus is able to oviposit, and its embryos complete development, in all sympatric unionid mussel species tested to date. Given a choice of mussels, however, they actively discriminate among them in making oviposition decisions (Smith et al., 2004). Two Unio and two Anodonta mussel species commonly co-occur with R. amarus in Europe. Rhodeus amarus show consistent preferences for Unio pictorum (L.), Unio tumidus Phillipson and Anodonta anatina (L.) over Anodonta cygnea (L.) throughout their European range.
Rhodeus amarus also prefer to spawn in mussels that do not already contain high numbers of R. amarus embryos (Smith et al., 2000a). A laboratory study demonstrated that these choices were adaptive; embryo mortality in mussels was strongly density-dependent and the strength of density dependence was significantly higher in A. cygnea (Smith et al., 2000b).

These results indicate that oviposition choice in R. amarus depends on variation in some aspect of host quality that females can discriminate (Mills & Reynolds, 2003; Smith et al., 2004). The oxygen content of water leaving the exhalant siphon has been shown as the most likely proximate cue for oviposition choice by R. amarus. Oxygen is the main factor affecting the embryo development; reduced oxygen retards embryo development and causes mortalities (Davis, 1975; Kamler, 1992; Smith et al., 2004). Anodonta cygnea exhibits significantly lower oxygen concentrations at their exhalant siphons than the other three European species and oxygen content in exhaled water decreases with the number of R. amarus embryos hosted by a mussel (Smith et al., 2001).

Host mussels may also eject bitterling eggs or developing embryos, either immediately (within a few seconds following oviposition) or later during embryo development (Mills & Reynolds, 2002). To dislodge bitterling eggs or embryos, mussels rapidly contract their valves and expel a stream of water. Mills & Reynolds (2002) showed that embryo ejection by European mussels matched the preference rank of R. amarus, with A. cygnea ejecting more embryos than the other three species. Embryos were typically ejected 4 to 6 days after oviposition, immediate ejections were rare (<5%), and host species ejected a total of 20 to 75% of oviposited embryos during development (Mills & Reynolds, 2002). Ejection of eggs by mussels has also been recorded in a related Asian bitterling species, the Chinese rose bitterling Rhodeus ocellatus ocellatus (Kner) (Kitamura, 2005). Adaptations shown by bitterling embryos for maintaining themselves in the gill chambers of mussels comprise a number of morphological and behavioural characters, including scaly tubercles on the egg and yolk-sac surface, a wing-like yolk sac, extensive embryonic vascularization and positive rheotaxis (Smith et al., 2004).

So far, it has been impossible to distinguish between alternative explanations of bitterling host preferences. Bitterling may make their oviposition choices with regard to host quality, based on (1) mussel physiological traits with no coevolutionary interaction (Smith et al., 2001; Mills et al., 2005), or (2) traits driven by coevolutionary dynamics between the parasite and host (Reynolds et al., 1997; Mills & Reynolds, 2002). At present, both hypotheses (termed ‘host quality’ and ‘coevolutionary dynamics’) are supported equally by correlations between host traits and R. amarus oviposition preferences. These hypotheses were tested by utilizing a fifth potential host species, Anodonta woodiana (Lea), which was recently introduced to Europe.

Studies involving species that have recently invaded outside their historical geographic range can provide crucial insights into host-parasite relationships (Cruz et al., 1998; Fraser et al., 2002; Lahti, 2005). Rhodeus amarus belongs to the subfamily Acheilognathinae, a group of cyprinid fishes comprising c. 40 species that all share the same reproductive mode (Arai, 1988). All bitterling fishes are restricted to Eastern Asia, except for R. amarus, a sister species of Rhodeus sericeus.
(Pallas) which occurs in the River Amur system, Sakhalin Island and rivers emptying into Peter the Great Bay and the Sea of Japan (Holčík, 1999). *Rhodeus sericeus* and *R. amarus* were considered conspecifics until recently (Bohlen et al., 2006). The divergence of these species on the basis of molecular data and application of the phylogenetic species concept suggest that they are genetically distinct species, though morphologically similar (Bohlen et al., 2006). *Rhodeus amarus* is distributed in Europe and Asia Minor and there is some evidence that two additional species or sub-species might occur at the periphery of its range: *Rhodeus colchicus* Bogutskaya & Komlev from the central Caucasus region and *Rhodeus meridionalis* Karaman from the River Vardar in Greece. The occurrence of bitterling in Central Europe is recent, with a large-scale molecular study indicating that *R. amarus* underwent a recent rapid expansion from the Pontic region (Bohlen et al., 2006). Although an absolute dating of this event is not yet possible, an unpublished bibliographical study (D. Van Damme & N. Bogutskaya, pers. comm.) identifies the *R. amarus* expansion as coinciding with the end of the Little Ice Age (c. 1850 AD). Regardless of the absolute timing of the expansion of *R. amarus* in Central and West Europe, the mussel populations used by *R. amarus* for spawning in Central Europe are hosts with a short sympatry compared with bitterling in Asia using Asian mussels that share a long sympatry with several bitterling species (Tomoda et al., 1977; Arai & Akai, 1988; Bohlen et al., 2006; Reichard et al., 2006).

In the present study, the responses of *R. amarus* to European mussel species were compared with responses to an Asian mussel, *A. woodiana*. *Anodonta woodiana* is widespread in East Asia (from the River Amur basin in the north to the subtropics) where it is a common host of several bitterling species, including *R. sericeus* (Hirai, 1965; Nagata, 1985; Holčík, 1999). Recently (1980s–1990s), *A. woodiana* was introduced into Central Europe (Beran, 1997; Watters, 1997), within the European geographical range of *R. amarus*. Consequently, *A. woodiana* was used to test whether *R. amarus* base their host choice upon mussel quality expressed by physiological traits irrespective of a coevolutionary relationship (Smith et al., 2001; Mills et al., 2005), or whether the coevolutionary dynamics and underlying mussel defence mechanisms (Reynolds et al., 1997; Mills & Reynolds, 2002) might play a significant role. In case of evidence for coevolution, the question addressed was which model of coevolution best characterizes the current stage of the bitterling-mussel relationship in Europe. It was predicted that the coevolutionary responses of European mussels to bitterling oviposition might be different to those of Asian mussels that have had a longer sympatric association with bitterling fishes, because European mussels represent relatively bitterling-naïve hosts.

To address these questions, oxygen consumption by *A. woodiana* was measured to assess its quality as a host for developing European bitterling embryos. Results were compared with those for European mussels. Second, oviposition preferences by *R. amarus* were tested, and the immediate, short-term, and long-term responses of five mussel hosts (four European, one Asian) to *R. amarus* oviposition was monitored in three independent experiments.

As evidence for the host quality hypothesis, it was predicted that *R. amarus* would prefer the mussel species with the lowest oxygen consumption rates, while avoiding mussels with the highest oxygen consumption rates (Smith et al., 2001), regardless of host geographic origin. As evidence for coevolution, the following predictions were made. For data to support the evolutionary lag model of
coevolution, it was predicted that *R. amarus* using Asian mussels would suffer a lower reproductive success than when using European, bitterling-naïve, hosts. For evidence of an evolutionary equilibrium between *R. amarus* and European host mussels, it was predicted that the reproductive success of *R. amarus* using *A. woodiana* would be: (1) greater if the host response to bitterling parasitism is specific to particular bitterling species and populations, or (2) equal if the host defence responses are general and apply to several bitterling species.

**MATERIALS AND METHODS**

**MUSSEL OXYGEN CONSUMPTION**

To compare among mussel species in their quality as hosts for developing *R. amarus* embryos, mussel oxygen consumption rates were measured. The dissolved oxygen concentration was recorded at the inhalant and exhalant siphons of *A. anatina*, *A. cygnea* and *A. woodiana* that did not contain any *R. amarus* embryos. Specimens of *A. anatina* and *A. cygnea* for this, behaviour and the short-term experiments were collected from an oxbow lake near the River Kyjovka and transported to the Institute of Vertebrate Biology, Brno, Czech Republic, where experiments were conducted. *Rhodeus amarus* were present in the lake at low densities (Smith et al., 2000a). *Anodonta woodiana* were collected from a canal connecting the water-cooling system of the Konin electric power plant with Lake Łęknickie. *Anodonta woodiana* were introduced to the lake as larval glochidia attached to cyprinid species in the mid 1980s, and are now found throughout the Lake Konin system (Kraszewski & Zdanowski, 2001). Although the origin of this particular *Anodonta woodiana* population cannot be traced, it must have evolved in long sympatry with bitterling fishes, because the geographic ranges of *A. woodiana* and bitterling (Acheilognathinae) in China entirely overlap. *Anodonta woodiana* used in the experiment were 3–5 years old and probably represented an F1 or F2 generation (Afanasjev et al., 2001). *Rhodeus amarus* are not present in Lake Łęknickie (Wilkonska, 1994). After collection, *A. woodiana* were stored at a fish hatchery close to the collection site and later transported to the Institute of Vertebrate Biology. Prior to the start of the experiment all mussels were individually marked by scoring their shells and stored together in a 1000 l fibreglass container with a layer of sand substratum and abundant phytoplankton food.

For oxygen measurements, experimental mussels were placed singly in sand-filled flowerpots in an artificial pool used for the long- and short-term exposure experiments. During trials mussels were not exposed to *R. amarus*. After mussels started filtering at a steady rate, oxygen consumption was measured using an oxygen probe following an established protocol (Smith et al., 2001). The probe was placed by a snorkeller as near as possible (1–3 mm) to the tip of the siphon, but without making contact with it. During measurements, the probe was held over the inhalant siphon for 5 to 20 s and then over the exhalant siphon for the same period. Readings were recorded once a stable dissolved oxygen measurement was obtained. Four readings, separated by at least 2 min, were taken for each mussel. The oxygen probe was calibrated every 30 min. Measurements were made for 15 *A. anatina* (mean shell size 117 cm, range 107–127 cm), 15 *A. woodiana* (mean 121 cm, range 115–145 cm) and 14 *A. cygnea* (mean 129 cm, range 98–140 cm). Water temperature ranged between 22.1 and 24.7° C during measurements.

**BEHAVIOUR EXPERIMENT**

A behavioural choice experiment was conducted to compare the behavioural responses of *R. amarus* to mussels, and mussels to *R. amarus* oviposition. The same three *Anodonta* species (*A. anatina*, *A. cygnea* and *A. woodiana*) were tested as for the mussel oxygen consumption recordings. *Rhodeus amarus* preferences were recorded on the basis
of pre-oviposition behaviour and oviposition choices. The immediate reaction of mussels to European bitterling oviposition (acceptance or ejection of eggs) was also recorded. The experiment was conducted in a large outdoor concrete pool at the Institute of Vertebrate Biology in May 2003. The pool measured 12.4 by 6.0 m, water depth was 0.6 m and water visibility was >3 m. The pool was stocked with an experimental population of *R. amarus* (c. 200 fish, female biased sex ratio of c. 1:2) collected from the River Kyjovka. Three mussel species (*A. anatina, U. pictorum* and *U. tumidus*) are common in the River Kyjovka. *Anodonta woodiana*, however, has never been found in the River Kyjovka despite repeated sampling (Reichard, 2002). The experimental pool was furnished with large plastic plants to serve as refuges for fish. Mussels in sand-filled flowerpots were always present in the pool when experiments were not being conducted to ensure the experimental population of *R. amarus* remained reproductively active during the test period. Throughout the experiment fish in the pool were fed daily with frozen bloodworm and foraged on algae that grew on the walls of the pool.

Tests were performed in two large net enclosures (100 × 80 × 80 cm, 5 mm mesh-size) inside the experimental pool. Each enclosure contained two sand-filled flowerpots placed 20 cm apart to enable a single male *R. amarus* to guard both simultaneously. Each flowerpot contained a single mussel and mussels were presented as a pair consisting of either *A. anatina* and *A. woodiana* or *A. cygnea* and *A. woodiana* combinations. Mussels were matched for shell size to avoid any possible effect of mussel size on fish preference. A total of 11 replicates of each species pair were completed. Mussel location (left or right) and mussel species pair were defined according to a randomly predetermined order.

To begin a test, a haphazardly selected male was captured in the pool by a snorkeller using a hand-net, released in the enclosure and allowed to establish a territory around the mussels. A female in spawning condition (with her ovipositor extended) was placed in the enclosure inside a 2 l glass jar to facilitate male settlement. Once a male began courting the female, the jar containing the female was removed and a second female in spawning condition was gently released into the enclosure. A snorkeller recorded pre-oviposition behaviour and oviposition choices for either 30 min or until oviposition, whichever was sooner. If oviposition occurred, the time and mussel species were recorded and the response of the mussel in terms of egg acceptance or ejection was observed for an additional 2 min.

The behaviours recorded during tests were as follows: Male and female inspection of mussels: the fish orientates itself at an angle of c. 75° from horizontal and positions the tip of its snout close (1–5 mm) to the exhalant siphon of a mussel. This behaviour probably serves to assess mussel quality before oviposition (Smith et al., 2001, 2004). Male ejaculation: sperm is released with the fish in a typical body posture; sweeping forward and down quickly over the inhalant siphon of the mussel. This behaviour represents a male investment towards a particular oviposition site (Smith et al., 2002, 2003; Reichard et al., 2004). Male leading: the male guides a female towards a particular mussel while courting. This behaviour indicates male preference for an oviposition site (Smith et al., 2002). Female skimming: female makes contact with the mussel exhalant siphon with the base of her ovipositor, but without inserting her ovipositor into the mussel. This behaviour may serve in the assessment of mussel quality (Smith et al., 2001, 2004) or mate attraction (Smith & Reichard, 2005). Following behavioural observations, test fish were captured using a hand-net and placed in aquaria to avoid pseudoreplication.

**EJECTION RATES OF *A. WOODIANA* IN ASIA AND EUROPE**

Ejection rates of *A. woodiana* in response to the oviposition of *R. amarus* and a bitterling from within the natural geographic range of *A. woodiana*, the Chinese rose bitterling, were compared. *Rhodeus o. ocellatus* is the most common bitterling in Asia, and co-occurs with *A. woodiana* throughout most of its range (Lin, 1998). In April 2005 *A. woodiana* were collected from Lake Poyang in the River Yangtze basin in China and transported to the Institute of Hydrobiology, Chinese Academy of Sciences facilities in Wuhan. A pair of *R. o. ocellatus* (collected in a local pond in Wuhan) were placed in 100 l aquaria with a single *A. woodiana* and observed until a successful spawning
occurred. After spawning, the response of the mussels was monitored for a period of 2 min and the number of ejected eggs was recorded. After 2 min the valves of experimental mussels were gently prised open using a mussel opening device (Kitamura, 2005) and the number of eggs on the gills was counted. No mussel contained fish embryos prior to the experiment and no mussel or fish was used more than once during the experiment. In June 2005, the same experimental procedure was repeated with R. amarus from the River Kyjovka and A. woodiana from Lake Licheńskie in 75 l aquaria at the Institute of Vertebrate Biology facilities.

SHORT-TERM EXPOSURE EXPERIMENT

A short-term exposure experiment was undertaken to compare the number of embryos in each of the mussel species on a time scale (24 h) that excluded the effect of ejections later in embryo development. The number of embryos in mussels was determined by R. amarus oviposition preferences and host ejections that occurred immediately after oviposition. Only Anodonta species were used: Asian A. woodiana and European A. anatina and A. cygnea. The experiment was conducted in May 2003 in the same pool used for the behavioural exposure experiment using a group of R. amarus from the River Kyjovka.

Mussels were presented to R. amarus in the experimental pool in four arenas. Each arena consisted of a single A. anatina, A. cygnea and A. woodiana placed in sand-filled flowerpots. Mussels were exposed to R. amarus spawning for 24 h. After 24 h, the mussels were replaced with a new set of mussels. After removal from the pool, mussels were measured to the nearest 1 mm and the number of R. amarus embryos on their gills counted by dissecting mussels. A total of 14 arenas were tested between 28 and 31 May.

LONG-TERM EXPOSURE EXPERIMENT

The purpose of this experiment was to compare the number of R. amarus embryos hosted by Asian A. woodiana and European A. anatina, A. cygnea, U. pictorum and U. tumidus under field conditions. The number of embryos in the host mussels at the end of the experimental treatment was affected by both the oviposition preferences and host ejections. The experiment was conducted in Lake Kociolek, a small lake (2.8 ha) in central Poland (52°37’02’’ N; 18°28’42’’ E) where R. amarus are the most abundant fish. During previous surveys, two mussel species were encountered in the lake: A. anatina and U. pictorum (M. Przybylski, unpubl. data). The lake had abundant aquatic vegetation in the shallow margins and a soft sediment substratum. For tests, A. anatina, A. cygnea, U. pictorum and U. tumidus were collected by hand from the Sulejowski Reservoir, located on the Pilica River. Rhodeus amarus are absent from the Sulejowski Reservoir. Both R. amarus [drift of young fish after emergence from mussels (Reichard et al., 2002)] and host mussels [glochidia attached to fish for several weeks (Bauer & Wächtler, 2000)], however, have life stages that are prone to large-scale dispersal along river corridors and across the floodplain. Consequently, the current lack of R. amarus in the man-made Sulejowski Reservoir does not imply that its mussel populations were evolutionary naïve to R. amarus oviposition. Collected mussels were stored in the aquarium facilities of the University of Łódź for 16 days prior to experiment and then transported to Lake Kociolek. Anodonta woodiana were collected from Lake Licheńskie and stored at a fish hatchery close to the collection site for 6 days before being transported to Lake Kociolek.

In May 2003, all mussels (19 individuals of each of the five test species) were transported to Lake Kociolek to begin the experiment. All mussels were given individual marks by scoring their shells, and distributed among 19 spawning arenas around the lake margin in a water depth of 45 to 70 cm. Each arena contained flowerpots filled-with a mixture of sand and lake sediment into which a single specimen of each mussel-species was placed. The mussels remained in position, exposed to R. amarus spawning, for
14 days. A period of 14 days was shorter than the minimum period for which *R. amarus* embryos are incubated in mussels (Smith *et al.*, 2004), but sufficiently long to enable a series of spawnings to occur in mussels by multiple males and females (Reichard *et al.*, 2004). On 6 June 2003, the mussels were collected and transported to the University of Łódź for dissection. Seven mussels of the 95 used in the experiment were not recovered; one *A. anatina*, two *A. cygnea*, one *A. woodiana*, one *U. pictorum* and two *U. tumidus*. Mussels were measured to the nearest 1 mm.

**DATA ANALYSIS**

For the long-term exposure experiment the difference in the number of embryos on the gills of mussels was tested among species using a one-way ANCOVA with Tukey’s HSD post-hoc test and mussel shell size as covariate. A two-way ANCOVA, with mussel species (*A. anatina*, *A. cygnea*, *A. woodiana*) and date (28–31 May) as factors and shell size as covariate, was used to test for differences among number of *R. amarus* embryos in mussels after 24 h in the short-term exposure experiment. In the behaviour experiment, all behaviours were expressed as a rate per hour. To compare fish behaviour and mussel preference, paired *t*-tests were used.

For estimates of mussel oxygen consumption, differences in the dissolved oxygen between inhalant and exhalant siphons were calculated for each pair of measurements and expressed as a proportional decline in dissolved oxygen concentration (Smith *et al.*, 2001). From a total of four replicates for each individual, the lowest value was removed and the mean calculated from the remaining three. Mussels occasionally stop filtration, even without disturbance. The removal of the lowest values increased the mean values of oxygen consumption while decreasing estimate variance. It is believed these gave the most accurate estimates of oxygen consumption. Including all four oxygen measurements, however, made no difference to the outcome of statistical tests. Non-parametric equivalents of tests were used where data were not normally distributed and did not respond to transformation.

**RESULTS**

**HOST QUALITY: MUSSEL OXYGEN CONSUMPTION**

The Asian *A. woodiana* appeared to represent a host of European bitterling embryos of potentially superior quality compared with *A. cygnea* and *A. anatina*. The change in dissolved oxygen concentration between inhalant and exhalant siphons differed among the three *Anodonta* species tested (square-root transformation, ANCOVA, d.f. = 2,40, *P* < 0.001) and shell size was not a significant covariate (ANCOVA, d.f. = 1,40, *P* > 0.05). *Anodonta cygnea* and *A. anatina* consumed significantly more oxygen than *A. woodiana* (Tukey’s HSD tests, *P* > 0.05 for *A. anatina* and *A. cygnea* comparison, *P* = 0.01 for *A. anatina* and *A. woodiana*, and *P* = 0.01 *A. cygnea* and *A. woodiana* comparisons). The mean ± s.e. reduction in dissolved oxygen concentrations for *A. cygnea* was 21.5 ± 4.4%, for *A. anatina* 14.5 ± 4.1% and for *A. woodiana* 4.4 ± 0.9%.

**HOST PREFERENCE BASED ON PREOVIPOSITION BEHAVIOUR**

Both male and female *R. amarus* recognized all three *Anodonta* species as potential hosts. In tests in which a pair of *R. amarus* had a choice between European (either *A. anatina* or *A. cygnea*) and Asian (*A. woodiana*) mussels,
both males and females inspected all species readily. Of the five preference measures used, *R. amarus* showed a preference for *A. anatina* over *A. woodiana* in three (Table I); both males and females examined *A. anatina* siphons at a higher rate and male *R. amarus* released sperm into the inhalant siphon of *A. anatina* more often than into *A. woodiana*. No difference, however, was detected in male leading and female skimming behaviour between *A. anatina* and *A. woodiana* (Table I). *Rhodeus amarus* showed no preference for either *A. cygnea* or *A. woodiana* in pair-wise tests between these two species (Table I).

**OVIPOSITION CHOICE AND HOST EJECTIONS**

Of the 22 choice tests completed, fish laid eggs in 14 trials. *Rhodeus amarus* used all three *Anodonta* species for oviposition. This small sample size did not permit rigorous statistical testing among all species. No clear differences, however, were detected in oviposition preferences in paired tests between *A. anatina* (two ovipositions) and *A. woodiana* (four ovipositions), or *A. cygnea* (four ovipositions) and *A. woodiana* (four ovipositions). Eggs were ejected immediately (<2 s) by *A. woodiana* in five of the eight ovipositions that occurred with this species. No cases of immediate egg ejection were observed with either *A. anatina* or *A. cygnea*. When eggs were ejected by *A. woodiana*, it was not possible to determine whether the whole clutch or only a part was ejected, though several eggs (up to four) were clearly seen in some cases.

A further five ovipositions by *R. amarus* in *A. woodiana* were observed in the additional test of egg ejections. Of these, three resulted in immediate ejections of eggs, while two clutches were not ejected within 2 min following oviposition. All three ejections were of either a single or two eggs, suggesting that they were partial ejections only; average clutch size in *R. amarus* is four eggs (Smith et al., 2004). Pooling these data with data from the behaviour experiment gave a total of eight immediate ejections out of 13 oviposition events of *R. amarus* into *A. woodiana* (62%).

**TABLE 1. Rhodeus amarus** preference for European v. Asian mussel hosts. Results of pair-wise comparisons of behavioural choices are shown (n = 11 pair-wise comparisons for each combination tested)

<table>
<thead>
<tr>
<th>Anodonta woodiana v. Anodonta anatina</th>
<th>Statistical test used</th>
<th>P</th>
<th>Preferred host</th>
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<tr>
<td>Male inspection</td>
<td>paired <em>t</em>-test</td>
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<td><em>A. anatina</em></td>
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<tr>
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<table>
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<td>0.155</td>
<td></td>
</tr>
<tr>
<td>Female inspection</td>
<td>paired <em>t</em>-test</td>
<td>0.581</td>
<td></td>
</tr>
<tr>
<td>Male sperm release</td>
<td>paired <em>t</em>-test</td>
<td>0.314</td>
<td></td>
</tr>
<tr>
<td>Male leading</td>
<td>paired <em>t</em>-test</td>
<td>0.581</td>
<td></td>
</tr>
<tr>
<td>Female skimming</td>
<td>Wilcoxon ranked</td>
<td>0.124</td>
<td></td>
</tr>
</tbody>
</table>

*Cases where a log10 transformation was applied to data before analysis.

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To compare these data for European bitterling with their Asian congeners, seven oviposition events by an Asian bitterling species, *R. o. ocellatus*, using *A. woodiana* in China, where the two species are sympatric, were also observed. Immediate ejections were recorded in six cases (86%), with a complete clutch ejected in one case and partial clutches in five. The rate of immediate ejection of *R. amarus* embryos by *A. woodiana* was not statistically different from the ejection rate for *R. o. ocellatus* (*G*-test, d.f. = 1, $P > 0.05$).

SHORT-TERM EXPOSURE EXPERIMENT

Following exposure to *R. amarus* oviposition for 24 h, European *A. anatina* and *A. cygnea* contained more fish eggs than the Asian *A. woodiana* (square-root transformation, ANCOVA, d.f. = 2,29, $P < 0.001$). The number of eggs on the gills differed significantly among the three mussel species (Tukey’s HSD tests: $P < 0.001$ for *A. anatina* and *A. woodiana*, $P < 0.001$ for *A. cygnea* and *A. woodiana*, and $P > 0.05$ for *A. cygnea* and *A. anatina*; Fig. 1). Mussel shell size was not a significant covariate (ANOVA, d.f. = 1,29, $P > 0.05$) and a significant interaction between the effects of mussel species and date (ANOVA, d.f. = 6,29, $P < 0.05$), indicating that the difference in egg number among mussel species was most pronounced when the highest total number of eggs were laid.

LONG-TERM EXPOSURE EXPERIMENT

*Anodonta woodiana* was the most inferior host for *R. amarus* embryo development. The total number of eggs and embryos on the gills of mussels exposed to *R. amarus* spawning for 2 weeks differed significantly among the five mussel

![Fig. 1. Host mussel use in the short-term experiment. Mean ± s.e. number of *Rhodeus amarus* embryos found in three *Anodonta* species (AC, *Anodonta cygnea*; AA, *Anodonta anatina*; AW, *Anodonta woodiana*) after 24 h exposure to *R. amarus* spawning. Number of dissected mussels is shown above error bars.](image-url)
species (Kruskal–Wallis test, d.f. = 4.88, P < 0.001). Notably, no *R. amarus* embryos were found in the gills of *A. woodiana* while large numbers of embryos were removed from the other four mussel species (Fig. 2). Most embryos were found in *U. tumidus* and *U. pictorum* with lower numbers recovered from *A. anatina* and *A. cygnea*.

**DISCUSSION**

An Asian mussel species, *A. woodiana*, which has recently been introduced to Europe from Eastern Asia, was used to test alternative hypotheses on mussel host preferences by European bitterling. The data support an hypothesis of coevolutionary dynamics between bitterlings and mussels (Mills & Reynolds, 2002), rather than an alternative hypothesis that associates bitterling preferences with host quality based on oxygen conditions within the mussel gills (Mills *et al.*, 2005). The oxygen conditions inside the gill cavity of *A. woodiana* were more suitable for embryo development than the conditions in the gills of European *Anodonta* hosts. A prediction of the host quality hypothesis is that *A. woodiana* would be the preferred host if European bitterling oviposition choice is determined solely by oxygen availability. Contrary to this prediction, *R. amarus* did not show a significant preference for *A. woodiana* over *A. anatina* and *A. cygnea*. It should be noted that in a previous study by Smith *et al.* (2001) estimates of mean oxygen consumption rates for *A. anatina* were lower (c. 7% compared to 14.5% in the present study), while for *A. cygnea*, similar values were obtained in both studies (c. 18 and 21.5%, respectively). Both these

![Fig. 2. Host mussel use in the long-term experiment. Mean ± s.e. total number of embryos in five mussel species (UP, Unio pictorum; UT, Unio tumidus; AC, Anodonta cygnea; AA, Anodonta anatina; AW, Anodonta woodiana) exposed to Rhodeus amarus spawning for 2 weeks. Number of dissected mussels is shown above error bars.](image-url)
estimates for A. anatina, however, lend equal support to the conclusions of the present study.

Although A. woodiana was not the preferred host, it was readily used for oviposition by R. amarus. Rhodeus amarus that deposited their eggs on the gills of A. woodiana, however, suffered a dramatic loss of reproductive success compared to R. amarus ovipositing into European mussels, which is consistent with the predictions for the evolutionary lag hypothesis. In 62% of observed oviposition events, A. woodiana immediately ejected at least a portion of the clutch laid by R. amarus. After 24 h few R. amarus eggs remained inside the gill cavities of A. woodiana (Fig. 1), most having been ejected. No R. amarus embryos were found in A. woodiana after 2 weeks (Fig. 2). In contrast, no immediate ejections by European mussels were detected in the present study and all European mussels contained some R. amarus embryos after 2 weeks (Fig. 2). These results do not lend support to the evolutionary equilibrium hypothesis. For an evolutionary equilibrium it was predicted that the reproductive success of R. amarus using A. woodiana would be greater, or equal, compared to that of R. amarus using European mussels (given that mussel response to bitterling parasitism is, or is not, specific for individual bitterling species, respectively).

Further, Reichard et al. (2006) found that European mussels suffer a dramatic reduction in growth when parasitized by R. amarus, in contrast to A. woodiana that were able to avoid this cost by egg and embryo ejections, showing that European mussels, as recent hosts of bitterling, have not had sufficient time to evolve defences to bitterling parasitism, in contrast to Asian A. woodiana (Reichard et al., 2006). Therefore, in the context of bitterling-mussel coevolution, the experimental data point toward an evolutionary lag in the relationship between R. amarus and European mussels. The ejection response of Asian A. woodiana, which share a long sympatry with several bitterling species of at least 16 million years (Tomoda et al., 1977) was considerably more pronounced than that of European mussels without a long evolutionary history with bitterling.

There have been earlier attempts to find evidence for an evolutionary lag (Mills & Reynolds, 2002) and evolutionary equilibrium (Mills et al., 2005) in the bitterling-mussel system, though none were conclusive. Mills & Reynolds (2002) studied ejections of U. pictorum, U. tumidus, A. anatina and A. cygnea in England, where R. amarus was introduced in the 20th century, and compared them with the ejection rates of the same mussel species from the present study sites in the Czech Republic (Smith et al., 2000a). Based on the observation that the two systems did not differ in the host response, they rejected the evolutionary lag model (Mills & Reynolds, 2002). In a further study, Mills et al. (2005) studied the costs to mussels of ejecting R. amarus eggs and found no support for an evolutionary equilibrium in the association. The results of these studies led to the conclusion that differences in mussel physiology among host species are most likely to be responsible for the differences in ejection rates among European mussels, with the width of water tubes (segments of gills where R. amarus embryos are wedged), ventilation rate, and the position of bitterling embryos inside the mussel gills as traits likely to explain differences in ejection rates among European mussel species (Mills et al., 2005).

These former studies, however, were based on the premise that R. amarus and their mussel hosts in the Czech Republic are of ancient sympathy compared
to a recent bitterling-mussel syzygy in England. *Rhodeus amarus* and its endemic close-relatives (*R. colchicus* and *R. meridionalis*) are the only European bitterling species (subfamily Acheilognathinae). All other bitterling species (c. 40) are confined to the area east of the River Mekong and Lake Baikal, in Laos, Vietnam, eastern China, Japan, Korea, and south-east Russia. These areas are separated by a distance of c. 4000 km (Holčík & Jedlička, 1994). Although the appearance of *R. amarus* in Europe is not unambiguously resolved, it is clear that East Asia is the centre of origin of the bitterling group (Arai, 1988; Okazaki *et al.*, 2001) and the occurrence of *R. amarus* outside this range is very recent (Bohlen *et al.*, 2006). Notwithstanding the absolute dating of European bitterling and mussel syzygy, the association in Asia is unquestionably considerably older (Tomoda *et al.*, 1977; Arai & Akai, 1988). Consequently, the lack of any evidence for coevolutionary interactions in former studies (Mills & Reynolds, 2002; Mills *et al.*, 2005) may have arisen because the authors compared two systems both with relatively recent syzygy. The present study made contrasts over a much larger evolutionary time scale and thereby permits the nature of the coevolutionary association to be demonstrated.

The evolutionary lag hypothesis infers that a parasite and its hosts are continually coevolving, generating cycles of adaptation and counter adaptation. If parasite prevalence or other factors differ among host populations, such populations may be at different phases of their cycles, forming a geographic mosaic of coevolutionary hotspots and coldspots (Thompson & Cunningham, 2002), with the scale of such structure depending on the dispersal abilities of parasite and host and metapopulation dynamics. Nevertheless, all bitterling-mussel systems studied in Europe to date (Czech Republic, Poland, Slovakia and U.K.) have revealed consistent host responses (Balon, 1962; Smith *et al.*, 2000b, 2004; Mills & Reynolds, 2002). Indeed, this homogeneity in response may be a consequence of the recent syzygy of *R. amarus* with all their mussel hosts in these parts of its European range where hosts have yet to evolve counter adaptations. The data from two *R. amarus* populations (Polish and Czech, from different river drainages) used in the present study lend support to the evolutionary lag hypothesis; all populations of European mussel hosts showed a weak response to *R. amarus* oviposition compared to an Asian mussel that has a long syzygy with bitterling. To obtain additional insights into the nature of the coevolutionary relationship it would be useful to compare these systems with the *R. amarus*–mussel association in the Pontic region, where the two groups share a much longer syzygy.

Oviposition preferences and embryo performances may vary between co-evolved and novel host-parasite relationships due to different host responses to closely related parasite species when host response is species-specific. The present study demonstrates that *A. woodiana* show a strong egg ejection response, not only to oviposition by *R. amarus*, but also to the sympatric Asian bitterling, *R. o. ocellatus*. This result suggests that mussel responses may not be strictly species-specific. Given the high ejection rate of bitterling eggs, *R. o. ocellatus* and other Asian bitterling species may show preferences for different hosts in central China where it is sympatric with over 25 mussel species (Wu, 1998) in relation to egg ejection rates. Comprehensive data on the host preferences of *R. o. ocellatus* over its native range have yet to be collected and it
cannot be judged whether this species make adaptive oviposition decisions linked to mussel egg ejection rates.

The bitterling–mussel association is analogous to that between avian brood parasites and their hosts in which parasitic species lay their eggs in the nests of other species, obtaining care from the foster parents (Payne, 1997). In the case of the common cuckoo *Cuculus canorus* L. in the U.K., it appears that hosts such as the dunnock *Prunella modularis* (L.) which are recent hosts of *C. canorus*, are most susceptible to parasitism (Davies, 1992). In contrast, hosts such as the reed warbler *Acrocephalus scirpaceus* Hermann, which have a long coevolutionary association with *C. canorus* in the U.K., have evolved numerous defences, including attacking *C. canorus* (Davies & Brooke, 1988), nesting in sites with a low probability of being parasitized by *C. canorus* (Öien et al., 1996), and being better able to discriminate between their own eggs and those of *C. canorus* (Davies & Brooke, 1989). In Central Europe, blackcaps *Sylvia atricapilla* L. are hosts with a long sympatry with *C. canorus* and have also evolved strong defences against *C. canorus* oviposition (Honza et al., 2004). As a result, *C. canorus* that historically specialized in parasitizing *S. atricapilla* (the blackcap gent) have apparently switched their oviposition preference to different host species (Honza et al., 2001), mainly *Acrocephalus* spp. warblers, which increased in abundance following the start of widespread carp *Cyprinus carpio* L. cultivation in the 16th century when the extent of fish pond associated reed beds increased dramatically (Honza et al., 2001). The same process might give rise to similar cycles of host switching in the bitterling-mussel association. Lahti (2005) showed that host defence mechanisms (egg mimicking) of the African village weaverbird *Ploceus cucullatus* Muller, which is parasitized by the Diederick cuckoo *Chrysococcyx caprius* Boddaert within their native range were significantly relaxed after only 100 and 200 years following two independent introductions to islands devoid of the brood parasite. The bitterling-mussel association in Europe may show comparable dynamics. Changes in the distribution of *R. amarus* from refugia in south-east Europe (Bohlen et al., 2006) since the last ice-age may be associated with temperature shifts, which have shown a cyclical pattern of change (Hewitt, 2004). Consequently, the bitterling–mussel association may have followed similar cycles of relaxation and reinforcement of host responses. Given that the fitness cost to mussels that host bitterling eggs and embryos are much lower than these that suffer avian host from cuckoos (Payne, 1997; Reichard et al., 2006), host response may take longer to spread among host populations.

A shortcoming of the present study is that it is based on data from a single host species with a long sympatry with bitterling fishes. Nevertheless, this additional host species allowed independent testing among different hypotheses on the nature of the associations between bitterling and mussels, which were otherwise impossible to resolve with only the four European host species. A more conclusive test would combine the use several bitterling and mussel species from areas of long, recent and no sympatry, and this will be the focus of ongoing studies.

Financial support came from the Leverhulme Trust and GAAV (600930501). HL acknowledges additional support from NSFC (grant 30470237) and MP was supported by Polish State Committee Scientific Research award (2P04F 01529). MR and CS
conceived and designed the work, MR ran experiments, analysed data and drafted the paper, MP provided and dissected mussels in Poland, MP and PK participated in experimental work, and HL oversaw the work in China and provided fish and mussels. We thank A. Kapusta and B. Zdanowski for collection of A. woodiana, M. Ondračková for help in the field, and N. Bogutskaya, M. Honza, J. Kitamura, S. Le Comber, E. Mallon, A. Naseka and anonymous referees for their valuable comments. Experiments complied with the laws in the countries in which they were conducted.

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