The co-evolutionary relationship between bitterling fishes and freshwater mussels: insights from interspecific comparisons

Martin Reichard,¹,²* Huanzhang Liu³ and Carl Smith¹

¹Department of Biology, University of Leicester, University Road, Leicester LE1 7RH, UK, ²Institute of Vertebrate Biology, Academy of Sciences of the Czech Republic, Květná 8, 603 65 Brno, Czech Republic and ³Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, People’s Republic of China

ABSTRACT

Hypothesis: In parasites that use hosts for offspring development, adults may base oviposition decisions on a range of host traits related either to host quality or the co-evolutionary relationship between parasite and host. We examined whether host quality or co-evolutionary dynamics drive the use of hosts in the bitterling–mussel relationship.

Organisms: Six species of bitterling fish (Acheilognathinae) and eight species of freshwater mussels (Unionidae, Corbiculidae) that are used by bitterling for oviposition.

Site of experiments: Experimental tanks in Wuhan, China, at the site of the natural distribution of the studied species.

Methods: Three experiments that controlled for host accessibility and interspecific interactions were conducted to identify host preferences among bitterling fishes and their mussel hosts. We started with a broad interspecific comparison. We then tested bitterling behavioural choices, their temporal stability, and mussel host ejection behaviour of the eggs of generalist and specialist bitterling species. Finally, we measured host mussel quality based on respiration rate and used published studies on mussel gill structure to infer mussel suitability as hosts for bitterling eggs.

Results: We found significant interspecific differences among bitterling species in their use of mussel hosts. Bitterling species varied in their level of host specificity and identity of preferred hosts. Host preferences were flexible even among apparently specialized species and fishes switched their preferences adaptively when the quality of individuals of preferred host species declined. Mussels varied considerably in their response to oviposition through egg ejections. Host preference by a generalist bitterling species correlated positively with host quality measured as the efficiency of the mussel gills to extract oxygen from inhaled water. Host ability to eject bitterling eggs correlated positively with their relative respiration rate, probably due to a higher velocity of water circulating in the mussel gill chamber.

Keywords: brood parasitism, co-evolution, egg ejection, host–parasite relationship, mutualism, oviposition, specialization, symbiosis.

* Address all correspondence to M. Reichard, Institute of Vertebrate Biology, Academy of Sciences of the Czech Republic, Květná 8, 603 65 Brno, Czech Republic. e-mail: reichard@brno.cas.cz

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INTRODUCTION

In many taxa, the choice of sites for oviposition is subject to strong selection, since it has significant consequences for offspring survival and performance. In parasites that use hosts for oviposition, a consequence is specific host preference, which may arise through differential host physiological suitability for offspring development (host suitability) or ecological factors responsible for host accessibility (encounter rate). Host specificity is typically driven by host preference, which can be fixed (if a preferred host is not found, no oviposition occurs) or flexible (non-preferred hosts are accepted when the preferred hosts are not available) (Craig et al., 1989). For example, the larvae of many phytophagous insects can complete development on several host plants, though they prefer certain hosts over others and there is variation in the strength of preference among species (Chew, 1977; Jaenike, 1978; Bossart, 2003).

Specialization for specific hosts can arise either with or without apparent co-evolutionary dynamics between parasite and host (Davies, 1992; Lapchin and Guillemaud, 2005). Adaptive responses by hosts may be lacking, especially when the cost, probability or intensity of an interaction is small (Jokela et al., 2000; Lapchin and Guillemaud, 2005). However, many interspecific relationships that incur significant costs to at least one partner typically result in a rapid co-evolutionary arms race between species when an adaptation in one partner is followed by reciprocal counter-adaptations in the other (Dawkins and Krebs, 1979). Such co-evolutionary dynamics may temporarily reach evolutionary equilibria (Takasu et al., 1993), which should promote host specialization, but which can also give rise to host switching when hosts evolve an effective response (Davies, 1992; Honza et al., 2001).

Bitterling (subfamily Acheilognathinae) are small cyprinid fishes with a distinctive spawning relationship with freshwater mussels. The bitterling–mussel association has proved valuable in clarifying several central questions in behavioural and evolutionary ecology (reviewed in Mills and Reynolds, 2003; Smith et al., 2004), including host use and host selectivity (Smith et al., 2000a, 2000b, 2001; Mills and Reynolds, 2002a, 2002b; Kitamura, 2005, 2006a, 2006b; Mills et al., 2005; Reichard et al., 2006, 2007). During the reproductive season, males defend territories around living mussels. Female bitterling develop long ovipositors that they use to place their eggs onto the gills of a mussel through the mussel’s exhalant siphon. 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. Eggs hatch in 36 h and developing embryos typically reside inside the mussel for 3–6 weeks before they actively depart from their host. Bitterling obtain no nourishment from their hosts and mussels serve solely as shelters for developing embryos. However, bitterling embryos can inflict significant fitness costs on mussels (Reichard et al., 2006) through damage to gill epithelium (Stadnichenko and Stadnichenko 1980), competing with mussels for oxygen (Smith et al., 2001), and disrupting water circulation over the mussel’s gills (Stadnichenko and Stadnichenko, 1980; Mills and Reynolds, 2003), with the result that mussel growth rates are compromised (Reichard et al., 2006).

There are approximately 40 bitterling species, separated into three genera (Acheilognathus, Rhodeus, Tanakia). Most species are distributed in eastern Asia (east of the Mekong River and Lake Baikal; in Laos, Vietnam, China, Japan, Korea, and south-eastern Russia). Only one species-complex is known from outside this area, Rhodeus amarus sensu lato [formerly known as European populations of the Asian species R. sericeus (Bohlen et al., 2006); R. amarus sensu stricto with a European-wide distribution, R. colchicus recently described from the periphery of R. amarus’ range in the Caucasian region (Bogutskaya and Komlev, 2001), and
an endemic population from the River Vardar in Greece, which has been raised to the level of species as *R. meridionalis* by Bohlen et al. (2006).

Bitterling are known to use a range of mussel species for oviposition. Their typical hosts are from the family Unionidae, although eggs have also been found in two species of Margaritiferidae (Zhul’kov and Nikiforov, 1988; Smith and Hartel, 1999). Unionids are the dominant freshwater mussel taxon in the regions of Eurasia occupied by bitterling, although they are also sympatric with other freshwater mussels (e.g. Corbiculidae, Sphaeriidae, Dreissenidae). Unionid mussels live buried in benthic sediments and filter the surrounding water to obtain food and oxygen. Unionid mussels are distributed worldwide. The East Asian unionids are grouped into three subfamilies – the Ableminae, Anodontinae, and Unioninae (Huang et al., 2002) – which differ in the anatomical structure and complexity of their gills.

Most studies on the bitterling–mussel relationship have largely concentrated on two bitterling species, *R. ocellatus* and *R. amarus*. European *R. amarus* is sympatric with four common unionid mussels and is able to oviposit, and its embryos complete development, in all of them. However, given a choice they actively discriminate among them and show consistent preferences throughout their European range (Balon, 1962; Smith et al., 2000a; Mills and Reynolds, 2002b). These choices are adaptive, as embryo mortality in mussels is significantly lower in preferred host species (Smith et al., 2000a). However, *R. amarus* choices are flexible, since they prefer to spawn in mussels that do not contain high numbers of bitterling embryos (Smith et al., 2000b), because embryo mortality in mussels is also strongly density-dependent (Smith et al., 2000a). Accordingly, *R. amarus* switch host species preferences when the quality of an individual of a preferred species deteriorates (Smith et al., 2000a) or is generally low (Mills and Reynolds, 2002b).

Oviposition choice in *R. amarus* depends on variation in host quality that females can detect. The oxygen content of water leaving the exhalant siphon (Smith et al., 2001) and flow rate of water leaving the exhalant siphon (Mills and Reynolds, 2002a) have been proposed as possible proximate cues for oviposition choice, although further studies are needed to clarify which cues bitterling use. Furthermore, evidence for co-evolutionary responses by mussels to bitterling oviposition has also recently been demonstrated (Reichard et al., 2006).

Mussels can eject developing bitterling embryos. To dislodge bitterling eggs or embryos, mussels rapidly contract their valves and expel a stream of water. Egg and embryo ejections typically occur within the first 6 days of embryonic development (Mills and Reynolds, 2002b; Kitamura, 2005). Oviposition preference of *R. amarus* matches the rank of host ejections: preferred hosts were found to eject the lowest number of developing embryos (Mills and Reynolds, 2002b). Although immediate ejections (< 2 min after oviposition) are rare in European mussels (Mills and Reynolds, 2002b; Smith et al., 2004), direct observations of bitterling oviposition has revealed that an Asian mussel, *Anodonta woodiana*, often ejects bitterling eggs immediately after oviposition (Reichard et al., 2007).

Non-random host use does not necessarily involve selective host choice. If encounter rate differs among host species, the cost of finding the most suitable host could outweigh the benefits of increased offspring survival. Furthermore, if several species compete for oviposition sites, species with less ability to defend such sites are expected to use less favourable resources. Studies on interspecific comparisons of host use by different bitterling species have so far been restricted to field surveys. Kondo et al. (1984) reviewed host use by five Japanese bitterling species. Eggs and embryos of *Tanakia lanceolata*, *T. tabira*, *T. limbata*, *Acheilognathus rhombeus*, and *Rhodeus sinensis* were found in up to seven mussel species from four genera (*Pseudodon, Inversidens, Anodonta*, and *Unio*), but were not
randomly distributed among host species. For example, in a small creek in Okayama Prefecture where five bitterling species co-occurred, five (of eight) mussel species were used as hosts. Bitterling species differed in the number of host species used (range of one to five), and thus appeared to show different levels of specialization (Kondo et al., 1984). A similar bitterling assembly in the River Harai, in Mie Prefecture, Japan varied slightly in host use by bitterling compared with the study by Kondo et al. (1984), and Kitamura (2006b) discussed the differences in the context of interspecific competition for mussels, mussel availability, and mussel use overlap. In contrast to studies from Japan (Hirai, 1965; Kondo et al., 1984; Kitamura, 2006b) that have found relatively plastic mussel use, Liu et al. (2006) identified embryos of three bitterling species in a sample of 16 mussel species from Lake Qinglan, China. Each bitterling species was found only in mussels from a single genus and only three mussel species were confirmed as hosts of bitterling (Liu et al., 2006).

These field survey data have limited power to detect host preference because they do not control for host accessibility (e.g. abundance, habitat use) or interspecific interactions between bitterling species; observed patterns of host utilization can arise from asymmetries in competitive ability among bitterling species. All bitterling males establish territories around mussels and defend them aggressively against rivals, including males of different bitterling species (M. Reichard, personal observation). If certain mussels serve as hosts of superior quality for all bitterling species, perhaps as a consequence of favourable physiological, anatomical or behavioural features, and are relatively scarce, competitive exclusion may be responsible for observed patterns of interspecific variation in host use. In the absence of interspecific competition and high abundance of a preferred host, all bitterling species would be predicted to make identical oviposition choices. Thus, interspecific competition could produce a pattern of apparent host specialization, with competitively inferior species able to use only unfavourable species when overall mussel abundance is low.

Except for the European R. amarus (which is allopatric in respect to other bitterling species), little is known about interspecific variation in host use in the absence of interspecific competition. Here we used a series of experiments to investigate mussel host use under controlled conditions. We excluded interspecific interactions among bitterling species and variation in host accessibility by using experimental tanks with single bitterling species and replicated sets of host mussels.

On the basis of field studies by Hirai (1965), Kondo et al. (1984), and Liu et al. (2006), and data on the bitterling–mussel relationship in Europe (Mills and Reynolds, 2003; Smith et al., 2004; Reichard et al., 2006), we made the following predictions:

1. The degree of host specificity varies among bitterling species – while some species are generalists, others may specialize on certain hosts.
2. Host preference may be detected in generalist species that use a broad range of hosts if hosts vary in quality.
3. Host preference is flexible and varies temporally in generalists as preferred hosts become filled with bitterling eggs, which decreases their quality as sites for embryo development.
4. Mussel gill structure, respiration rate, and egg ejection rate predict host quality and, therefore, preferences by bitterling.

To test these predictions, we conducted a series of three experiments. The first, a long-term study, provided a measure of the distribution of the eggs of several bitterling species among different mussel host species. The pattern of egg distribution represented the
outcome of oviposition decisions by bitterling and egg ejections by mussels. A second, short-term experiment concentrated on bitterling species identified from the first experiment as specialist and generalist, and examined host use before host responses occurred. The short-term experiment was complemented with a third experiment that examined the behaviour of a specialist and generalist bitterling species to hosts, and the immediate responses of host mussels to bitterling. We additionally measured mussel host quality in terms of respiration rate and used published studies on mussel gill structure to infer their suitability as hosts for bitterling eggs.

MATERIAL AND METHODS

Long-term experiment (Experiment 1)

The aim of this experiment was to compare the suitability of a range of mussel species to serve as hosts of different bitterling species. Bitterling were housed in single-species tanks and were offered six sets of eight mussel species for oviposition. After 15–20 days, all mussels were dissected and the numbers of bitterling eggs and embryos were counted. Therefore, the numbers of eggs and embryos in mussels were determined by bitterling oviposition preference as well as host ejections during the post-oviposition period. The choice of mussel and bitterling species reflected their abundance during field collection and included mussel species from all three unionid subfamilies (differing in gill anatomy) and one species of Corbiculidae (Table 1).

The experiment was carried out in April–May 2004 and 2005 at the Institute of Hydrobiology, Chinese Academy of Sciences in Wuhan (IHB). In 2004, five bitterling species were used: Rhodeus ocellatus, Rhodeus fangi, Acheilognathus chankaensis, Acheilognathus peihoensis, and Acheilognathus imberbis. In 2005, we also tested Rhodeus sinensis and repeated the experiment with R. fangi and A. imberbis (which laid relatively few eggs in 2004). Fish were collected from ponds, lakes, and irrigation channels in Hubei Province, transported to the laboratory, and immediately stocked into large (300-litre) aquaria. Six males and 18 females of each bitterling species were housed in experimental tanks (135 × 95 × 50 cm) in a dedicated experimental room. Only 12 females each of A. chankaensis and R. fangi, and five females of A. peihoensis, were available in 2004 and only these were tested with six males. All fish were sexually mature and reproductively active; males guarded territories around mussels and females had extended ovipositors.

Six plastic basins (30 cm diameter) filled with sand and gravel were placed inside each experimental tank. Each basin contained eight mussels: Acuticosta chinensis (Unioninae, gill type B), Anodonta globosula (Anodontinae, gill type D), Cuneopsis capitata (Unioninae, gill type C), Corbicula fluminea (family Corbiculidae, gill type E), Hyriopsis cummingi (Ableminae, gill type A), Lamprotula caveata (Ableminae, gill type A), Lanceolaria grayana (Unioninae, gill type C), and Unio douglasiae (Unioninae, gill type B). In 2005, C. capitata was not available, so instead we used Schistodermsus lampreyanus (Unioninae, gill type B). For A. globosula, we had only nine individuals available for this experiment in 2005. Therefore, only three A. globosula were used in each experimental tank, while the other three basins contained only the other seven mussel species being examined. In addition, we lacked four individuals of H. cummingi in 2004 in the experimental tank in which we tested A. peihoensis.
Initial results indicated that *Rhodeus ocellatus* to be a generalist in its use of mussels for oviposition and we wished to test the extent of its range of host use. Therefore, in 2004 an additional seven mussel species were exposed to spawning by *R. ocellatus* after the end of the initial experiment. The additional test mussel species were: *Aculamprotula tortuosa* (Ableminae, gill type A), *Arconaia lanceolaria* (Unioninae, gill type unknown), *Cristaria plicata* (Anodontinae, gill type D), *Cuneopsis pisciculus* (Unioninae, gill type C), *Lanceolaria eucylindrica* (Unioninae, gill type C), *Schistodesmus lampreyanus* (Unioninae, gill type B), and *Schistodesmus spinosus* (Unioninae, gill type B). The experimental protocol was identical to the main experiment and was repeated twice.

All experimental mussels were collected from Lake Qinglan, Jiangxi Province, in the catchment of the middle reaches of the River Yangtze, by commercial mussel fisherman using a bottom dredge. For mussel size and gill structure characteristics, see Table 1. Experimental animals experienced a natural day length. Fish were fed twice each day with live *Tubifex*, frozen chironomid larvae, and commercial flake food. Mussels were fed every 2–4 days with phytoplankton. The survival of fish and mussels was high. Fish and mussels were checked twice each day during feeding and any dead fish (a total of seven) were replaced. Dead mussels (a total of four) were replaced if found within the first 3 days of the start of the experiment, otherwise they were not substituted (\( n = 10 \) non-substituted

<table>
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<th>Species</th>
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<th>Gill type</th>
<th>Gill anatomy</th>
<th>Mean length</th>
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<tr>
<td><em>Lamprotula caveata</em></td>
<td>Ableminae</td>
<td>A</td>
<td>without true water tubes or septa</td>
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<td>1.1</td>
<td>46–78</td>
<td>45</td>
<td>Lca</td>
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<td><em>Hyriopsis cummingi</em></td>
<td>Ableminae</td>
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<td><em>Aceticosta chinensis</em></td>
<td>Unioninae</td>
<td>B</td>
<td>with water tubes and perforated septa</td>
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<td>0.6</td>
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<td><em>Schistodesmus lampreyanus</em></td>
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<td>0.7</td>
<td>36–47</td>
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<td>Sla</td>
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<td><em>Unio douglasiae</em> (Gray)</td>
<td>Unioninae</td>
<td>B</td>
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<td><em>Cuneopsis capitata</em></td>
<td>Unioninae</td>
<td>C</td>
<td>with water tubes and non-perforated septa</td>
<td>69</td>
<td>2.9</td>
<td>52–106</td>
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<td><em>Lanceolaria grayana</em></td>
<td>Unioninae</td>
<td>C</td>
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<td>1.3</td>
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<td><em>Corbicula fluminea</em></td>
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<td>21–38</td>
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*Note:* The taxonomic designation, gill type, mean, standard error (SE), and range of total shell length and mussel identification codes are given. Gill type in parentheses denotes uncertainty in designation.
mussels, 2.7%). Experimental tanks were aerated and water temperature varied with ambient from 21 to 25°C. During a spell of cold weather, thermostatic aquarium heaters were used to maintain the water temperature at 22°C to ensure that fish remained reproductively active. At completion, the experiment yielded 882 eggs distributed among 62 mussels (20% of 316 dissected mussels).

**Short-term experiment (Experiment 2)**

This experiment was used to compare host use over a shorter time (24 h) to exclude the effects of embryo mortality that may occur later in embryo development. Two bitterling species (*Rhodeus ocellatus* and *R. sinensis*), showing different levels of host specialization, were used with the four most common mussel species representing members of all three unionid subfamilies (*Lamprotula caveata*, *Lanceloaria grayana*, *Unio douglasiae*, *Anodonta globosula*). The number of embryos in mussels in this experiment was determined by bitterling oviposition preferences and host ejections that occurred immediately after oviposition.

The experiment was undertaken during May 2004 (*Rhodeus ocellatus*) and April and May 2005 (*R. sinensis*) using aquarium facilities at the IHB. A stock of approximately 100 fish (sex ratio 1:1) was housed in a large experimental tank (120 × 50 × 50 cm and 190 × 100 × 70 cm in 2004 and 2005, respectively). Fish experienced natural day length and were fed twice each day. Water was continuously aerated and filtered. A plastic sand-filled basin that contained four mussels (one *L. caveata*, *A. globosula*, *L. grayana*, and *U. douglasiae*) was placed in each aquarium for 24 h. After this time, the mussels were replaced with a new set of mussels. After removal from the tank, mussels were measured to the nearest 1 mm and the number of bitterling eggs on their gills was counted. Before the experiment, mussels were stored in large water containers without bitterling and fed with phytoplankton. The mussels were from the same source as Experiment 1. In total, this experiment yielded 322 eggs distributed among 28 mussels (70% of 40 dissected mussels) for *R. ocellatus* and 487 eggs distributed among 28 mussels (64% of 44 dissected mussels) for *R. sinensis*.

**Behavioural and oviposition experiment (Experiment 3)**

This experiment was used to compare: (1) oviposition preference and behavioural responses of *Rhodeus ocellatus* and *R. sinensis* towards four mussel species (*Lamprotula caveata*, *Anodonta globosula*, *Lanceloaria grayana*, and *Unio douglasiae*); (2) clutch size of *R. ocellatus* and *R. sinensis*; and (3) the behavioural responses of the four mussel species to bitterling oviposition. Direct behavioural observations were performed in aquaria, using a pair of fish and four mussels. Experimental aquaria measured 120 × 50 × 50 cm. The experiment was conducted between April and May 2004 and 2005 (for *R. ocellatus* and *R. sinensis*, respectively).

For each replicate, a male was haphazardly chosen from a stock of experimental fish and placed in an aquarium with four mussels in individual plastic cups. The male was allowed to settle and establish a territory for at least an hour. The mussels were covered with netting (to prevent oviposition), although the fish had visual and olfactory contact with the mussels. After one hour, a female in spawning condition (with her ovipositor fully extended) was gently released into the aquarium. The pair was allowed to settle for a minimum of 10 min.
When both fish began to show interest in the mussels (by inspecting the siphons), the mussels were uncovered and behavioural observation began.

Fish behaviour was observed for 40–45 min or until oviposition, whichever occurred first, using an established protocol (Reichard et al., 2004). Once a female oviposited, 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 mussel siphons**: the fish orientates itself at an angle of about 75° from horizontal and positions the tip of its snout close to the exhalant siphon of a mussel. This behaviour serves to assess mussel quality before oviposition (Smith et al., 2001).
- **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**: the female makes contact with the mussel exhalant siphon with the base of her ovipositor, but without inserting her ovipositor into the mussel. This behaviour serves in mate attraction (Smith and Reichard, 2005) and may indicate female preference for an oviposition site (Candolin and Reynolds, 2001).
- **Spawning**: the female inserts the base of her ovipositor into the exhalant siphon, the ovipositor unfurls into the mussel gill, and the eggs are ejected. This behaviour is an ultimate measure of female oviposition choice.

Following behavioural observations, test fish and mussels were removed from the experimental aquarium and were not used again. Fish and mussels were measured to the nearest 1 mm (fish standard length, mussel total shell length) and the number of bitterling eggs on the gills of mussels that received a batch of eggs was counted.

**Respirometry**

The measurement of mussel respiration rates was conducted to estimate interspecific differences in mussel physiology. Interspecific differences in the efficiency of oxygen consumption within the mussel gills correlate with oviposition host choice by *Rhodeus amarus* and the ejection rates of four European unionid hosts (Smith et al., 2001; Mills and Reynolds, 2002a, 2002b). The two measures are complementary – hosts with the highest flow rates at the exhalant siphon had the highest oxygen consumption rates when oxygen content was compared directly between water in the inhalant and exhalant siphons (Mills and Reynolds, 2002a; Smith et al., 2001, 2004). Furthermore, the interspecific rank of water flow rates at the exhalant siphon of a mussel correlated with *R. amarus* embryo ejection rates in European mussels – that is, the fastest flow rate matched the highest ejection rate (Mills and Reynolds, 2002b). We used respiration rate as a measure of host quality because it encapsulates both measurements in a single value and more accurately represents the conditions experienced by bitterling embryos.

To measure respiration rates, individual mussels were placed in 3-litre closed respirometry chambers for 8 h. The water temperature in chambers was 24.1–24.3°C, which matched the temperature experienced by mussels during the experiments. The change in oxygen content of the water over the 8-h period was measured by standard titration methods using MnSO₄ (Rowland and Grimshaw, 1989). A pilot study showed that 8 h was the optimum time interval over which the oxygen content in the respirometry chamber decreased substantially while remaining above the limits that might affect mussel respiration rates. Final oxygen
concentration in the chamber never fell below 4.5 mg·l⁻¹ (mean ± standard error: 6.5 ± 0.08 mg·l⁻¹). Mussel shell size (measured along the longest shell axis) and mussel dry mass (soft tissue dissected after experiment and dried at 65°C for 48 h) were measured for each mussel. Total respiration rate was the total reduction in oxygen per unit time. Relative respiration rate was the total respiration rate per unit dry weight of mussel tissue.

Data analysis
The mean number of developing embryos in the gill chambers of mussels and prevalence among mussels (proportion of mussels containing at least a single bitterling embryo) were calculated for each mussel species in Experiments 1 and 2. Analysis of variance (ANOVA) was used to compare the number of bitterling eggs in mussels, mussel respiration rates (total and relative), and pre-oviposition fish behaviour. Behavioural data were expressed as rates each hour. Data were tested for normality before analysis and transformed to meet test assumptions where necessary. The non-parametric Kruskal-Wallis test was used where data were not normally distributed and did not respond to transformation. Tukey HSD tests (parametric) or Wilcoxon tests (non-parametric) were used for pair-wise post-hoc comparisons. The skimming rate of female Rhodeus ocellatus and leading rate of male R. sinensis strongly deviated from normality and showed binomial distributions. Therefore, we separated cases based on whether fish performed these behaviours or not, and analysed the data using a G-test. Skimming behaviour by female R. sinensis was too infrequent to permit statistical testing. G-tests were also used to analyse oviposition preference and egg ejections in Experiment 3.

RESULTS

Long-term experiment (Experiment 1)
The six bitterling species tested varied in the total number of embryos recovered and their distribution among mussel species. Rhodeus ocellatus oviposited the highest number of eggs, which were distributed among all the unionid species presented to them. Rhodeus fangi embryos were also found in a wide range of hosts, but principally in Lamprotula caveata, Hyriopsis cummingi, and Acuticosta chinensis (Table 2). In contrast, embryos of Rhodeus sinensis and Acheilognathus peihoensis were found only in a single mussel species. While R. sinensis distributed their eggs among four of six Unio douglasiae mussels presented to them, A. peihoensis eggs were all found in a single Anodonta globosula mussel. All 189 A. peihoensis eggs were at the same developmental stage, although it is unclear whether all eggs came from a single oviposition event. Acheilognathus chankaensis used two host species, Lamprotula caveata (prevalence 50%) and U. douglasiae (prevalence 17%). We found no Acheilognathus imberbis embryo in mussels in 2004 and only three embryos (in U. douglasiae and H. cummingi) in 2005 (Table 2). No bitterling embryos were found on the gills of the corbiculid Corbicula fluminea. For statistical tests of non-random use of hosts, see Table 3.

In the additional test with R. ocellatus, embryos were found in all the unionid hosts tested, including Lanceolaria eucylindrica (36 and 20 embryos in the first and the second set of mussels, respectively), Arconaia lanceolaria (37 and 14 embryos), Schistodesmus lampreyanus (37 and 9 embryos), Schistodesmus spinosus (5 and 30 embryos), Cuneopsis
Table 2. Mean number (and standard error) of bitterling eggs and embryos in dissected mussels in the long-term experiment (Experiment 1), their prevalence (P, the proportion of mussels that contained at least one egg or embryo, in %), and the number of mussels recovered (N).

<table>
<thead>
<tr>
<th>Species</th>
<th>(a) 2004</th>
<th></th>
<th>Mean</th>
<th>SE</th>
<th>P</th>
<th>N</th>
<th>Mean</th>
<th>SE</th>
<th>P</th>
<th>N</th>
<th>Mean</th>
<th>SE</th>
<th>P</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lamprotula caveata</td>
<td>R. ocellatus (n = 366; 31)</td>
<td></td>
<td>3.3</td>
<td>1.6</td>
<td>50</td>
<td>6</td>
<td>1.5</td>
<td>1.1</td>
<td>33</td>
<td>6</td>
<td>12.5</td>
<td>6.0</td>
<td>50</td>
<td>6</td>
</tr>
<tr>
<td>Lanceolaria grayana</td>
<td>R. fangi (n = 23; 6)</td>
<td></td>
<td>4.2</td>
<td>2.4</td>
<td>50</td>
<td>6</td>
<td>0.7</td>
<td>0.5</td>
<td>33</td>
<td>6</td>
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<td>0.0</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Unio douglasiae</td>
<td>A. chankaensis (n = 78; 4)</td>
<td></td>
<td>10.7</td>
<td>3.0</td>
<td>100</td>
<td>6</td>
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<td>1.1</td>
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<tr>
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<td>A. peihoensis (n = 189; 1)</td>
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<td>16.3</td>
<td>5.3</td>
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<tr>
<td>Hyriopsis cummingi</td>
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<td>100</td>
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<tr>
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<td></td>
<td></td>
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<td>1.0</td>
<td>50</td>
<td>2</td>
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<td>6</td>
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<td>6</td>
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<tr>
<td>Corbicula fluminea</td>
<td></td>
<td></td>
<td>0.0</td>
<td>0.0</td>
<td>0</td>
<td>6</td>
<td>0.0</td>
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<td>6</td>
<td>0.0</td>
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<td>Anodonta globosa</td>
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<td>1</td>
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<tr>
<td>Hyriopsis cummingi</td>
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<td>0.0</td>
<td>0</td>
<td>6</td>
<td>7.7</td>
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<td>67</td>
<td>6</td>
<td>0.5</td>
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<td>Acuticosta (chinensis?)</td>
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<td></td>
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<td>5</td>
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<td>6</td>
<td>0.0</td>
<td>0.0</td>
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</tr>
</tbody>
</table>

Note: Total number of eggs and embryos and the number of mussels that contained at least one egg or embryo are indicated after bitterling species identity (n).
pisciculus (23 and 8 embryos), Cristaria plicata (31 and 0 embryos), and Aculamprotula tortuosa (9 embryos in the first set of mussels).

**Short-term experiment (Experiment 2)**

*Rhodeus ocellatus* eggs were distributed among all host species, although the number of eggs varied significantly among mussels (ANOVA: $F_{3,36} = 4.98$, $P = 0.005$). All *Anodonta globosula* individuals contained some eggs (prevalence 100%), although other mussel species were also frequently used: *Unio douglasiae* (prevalence 70%), *Lanceolaria grayana* (60%), and *Lamprotula caveata* (50%). *Anodonta globosula* contained more eggs than *U. douglasiae* and *L. caveata* (Tukey tests, Fig. 1a).

<table>
<thead>
<tr>
<th>Fish species, year</th>
<th>d.f.</th>
<th>$H$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Rhodeus ocellatus</em>, 2004</td>
<td>7,44</td>
<td>26.3</td>
<td>&lt;0.001</td>
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<tr>
<td><em>Rhodeus fangi</em>, 2004</td>
<td>7,48</td>
<td>11.2</td>
<td>0.132</td>
</tr>
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<td><em>Acheilognathus chankaensis</em>, 2004</td>
<td>7,48</td>
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<td>0.014</td>
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<td><em>Acheilognathus peihoensis</em>, 2004</td>
<td>6,37</td>
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<tr>
<td><em>Rhodeus sinensis</em>, 2005</td>
<td>7,43</td>
<td>26.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><em>Rhodeus fangi</em>, 2005</td>
<td>7,43</td>
<td>17.2</td>
<td>0.016</td>
</tr>
<tr>
<td><em>Acheilognathus imberbis</em>, 2005</td>
<td>7,48</td>
<td>6.1</td>
<td>0.525</td>
</tr>
</tbody>
</table>

*Note:* Statistical significance denotes a non-random distribution of bitterling eggs and embryos among host mussel species. For egg and embryo numbers in specific hosts, see Table 2.

**Fig. 1.** Mean number of (a) *Rhodeus ocellatus* and (b) *R. sinensis* eggs in four mussel species after 24 h exposure to bitterling spawning. Note that the two bitterling species were tested separately. Error bars represent one standard error. Letters above error bars denote significantly different groups determined by post-hoc pair-wise comparisons; values with the same letter did not differ significantly.
Rhodeus sinensis eggs were also found in all host species tested, although there was a significant difference among host species (Kruskal-Wallis test: $H_{3,44} = 9.54$, $P = 0.023$). Unio douglasiae was the most frequently used host (prevalence 82%) and contained the highest number of eggs (Fig. 1b), followed by L. caveata (prevalence 73%), L. grayana (55%), and A. globosula (46%).

**Behavioural and oviposition experiment (Experiment 3)**

Rhodeus ocellatus showed a spawning preference for Anodonta globosula, while Lamprotula caveata was used least often ($G$-test: $G = 10.29$, d.f. = 3, $n = 25$, $P = 0.001$; Fig. 2). Male R. ocellatus inspected the siphons of A. globosula most frequently, followed by Unio douglasiae, L. caveata, and Lanceolaria grayana (Kruskal-Wallis test: $H_{3,100} = 8.39$, $P = 0.039$; Fig. 3a). Male leading rate (Kruskal-Wallis test: $H_{3,100} = 3.57$, $P = 0.312$; Fig. 3b) and female inspection rate (Kruskal-Wallis test: $H_{3,100} = 2.69$, $P = 0.442$; Fig. 3c) did not differ among mussels. Female R. ocellatus skimmed over the siphons of Unio douglasiae and A. globosula more often than over L. grayana and L. caveata ($G$-test: $G = 9.14$, d.f. = 3, $P = 0.003$).

Of 20 replicates, Rhodeus sinensis oviposited only in six trials. All ovipositions were into Unio douglasiae. Overall, R. sinensis showed much more interest in Unio douglasiae mussels than in the other three mussel species. Both males (Kruskal-Wallis test: $H_{3,80} = 49.1$, $P < 0.001$) and females (ANOVA on log$_{10}$ transformed data: $F_{3,76} = 14.18$, $P < 0.001$) inspected siphons of Unio douglasiae most often (Figs. 3a, 3c). Male R. sinensis led females to Unio douglasiae more frequently than to the other mussels tested ($G$-test: $G = 8.81$, d.f. = 3, $P = 0.003$, Fig. 3b). Female R. sinensis skimmed only over Unio douglasiae (in 9 of 20 replicates) and A. globosula (single replicate).

**Clutch size and ejections**

Rhodeus ocellatus laid significantly smaller clutches than R. sinensis ($t$-test: $t_{18} = 6.59$, $P < 0.001$). The mean number of eggs in R. ocellatus was 4.4 (standard error = 1.3; $n = 14$) and in R. sinensis 15.7 (standard error = 2.6; $n = 6$).

![Fig. 2. Oviposition preference of (a) Rhodeus ocellatus and (b) R. sinensis females based on the number of clutches laid on different host species ($n = 25$ oviposition events for R. ocellatus and $n = 6$ for R. sinensis).](image-url)
Fig. 3. Pre-oviposition behaviour of (a) Rhodeus ocellatus and (b) R. sinensis: (a) male inspection of mussel exhalant siphon, (b) male leading of females towards mussel, and (c) female inspection of mussel exhalant siphons. Error bars represent one standard error. Letters above error bars denote significantly different groups as determined by post-hoc pair-wise comparisons; values with the same letter did not differ significantly.
Mussels differed in their response to *R. ocellatus* oviposition. Immediately after oviposition, *Lanceolaria grayana* ejected more eggs than the other three mussel species (*G*-test: *G* = 4.51, d.f. = 3, *P* = 0.034) and the overall frequency of ejection ranged from 18 to 91% of eggs laid in specific mussels (Fig. 4). Mussels ejected either an entire clutch or only part of a clutch (1–3 eggs), although the lack of quantitative data from some oviposition events does not enable quantitative comparison of the proportions ejected. Overall, ejections were most frequent from mussels with the most complex gill structure (*L. grayana*) and least frequent from *Lamprotula caveata* with the simplest gill structure (Fig. 4). In *R. sinensis*, partial clutch ejection was observed in all six oviposition events with *Unio douglasiae*.

**Mussel respiration**

The total respiration rate differed significantly among mussel species (ANOVA on log10 transformed data: $F_{3,64} = 3.76$, $P = 0.015$). *Lamprotula caveata* had the highest total respiration rate and *Anodonta globosula* the lowest (Tukey tests; Fig. 5a). Relative respiration rates also varied significantly among mussel species (ANOVA on log10 transformed data: $F_{3,64} = 12.73$, $P < 0.001$), being highest in *Lanceolaria grayana* and lowest in *L. caveata* (Tukey tests; Fig. 5b).

In *Rhodeus ocellatus*, for which a mussel preference could be estimated (Fig. 2), we observed a perfect match between mussel total respiration rate and *R. ocellatus* oviposition preference; the host with the lowest total respiration rate (*A. globosula*) was preferred, while *L. caveata*, with the highest total respiration rate, was used least often (Figs. 2 and 5).

There was a positive correlation between mussel relative respiration rate and mussel ejection rate of *R. ocellatus* eggs (Pearson correlation: $r = 0.956$, $n = 4$, $P = 0.044$). The rank order (highest to lowest relative respiration rate and egg ejection rate) was *L. grayana* > *U. douglasiae* > *A. globosula* > *L. caveata* (Figs. 4 and 5).

**DISCUSSION**

An understanding of host–parasite relationships and patterns of co-evolution can be greatly enhanced from studies involving interspecific comparisons (Tillberg, 2004; Lopez-Vaamonde...
The present study investigated interspecific differences in host use, host preference, and host responses to parasitism in an unusual symbiosis between bitterling fishes and unionid mussels that are used by bitterling for oviposition.

We found that the six bitterling test species differed markedly in their use of mussel hosts and, confirming our first prediction, there were interspecific differences in host specificity. In the long-term experiment, *Rhodues ocellatus* eggs and embryos were found in all unionid mussels tested, while eggs and embryos of *R. sinensis* and *Acheilognathus peihoensis* were found only in a single mussel species. Other species were intermediate between the two extremes, using a range of 2–5 unionid mussel species. While all unionid mussels were used by at least one bitterling species, no bitterling eggs or embryos were found in *Corbicula fluminea* from the family Corbiculidae (Table 2).

A preference for particular host species was detected even in *R. ocellatus*, supporting our second prediction that host preference can be detected even in generalist species. Notably, bitterling species varied in the identity of their preferred mussel species. A different level of host specificity and identity of preferred hosts has been reported from field-based studies (Hirai, 1965; Kondo *et al.*, 1984; Kitamura, 2006b; Liu *et al.*, 2006) and three possible explanations of the observed patterns were proposed: host preference, mussel accessibility, and competitive exclusion. We controlled for interspecific interactions among bitterling species and made all mussel species equally available for oviposition by placing them in the same location. Therefore, we can exclude host accessibility and competitive exclusion as explanations for species-specific host use.

Our data from the long-term experiment (Experiment 1) could not distinguish between host preference and host suitability. For example, bitterling species may have preferred the same host species, but egg and embryo mortality by asphyxiation (Smith *et al.*, 2000a; Kitamura, 2005) and mussel ejection of eggs and embryos (Mills and Reynolds, 2002b; Smith *et al.*, 2004; Kitamura, 2005; Reichard *et al.*, 2007) over a 2-week period resulted in an apparent host preference. A further complication in the long-term experiment relates to clutch size among bitterling species.

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**Fig. 5.** Physiological performance of the four mussel species used in all experiments: (a) total respiration rate (oxygen consumption over 8 h), (b) relative respiration rate (raw respiration rate expressed per gram of dry body mass). Error bars represent one standard error. Letters above error bars denote significantly different groups as determined by *post-hoc* pair-wise comparisons; values with the same letter did not differ significantly.
Whereas 366 eggs and embryos (representing at least 80 clutches) of *R. ocellatus* were recovered from mussels, 189 eggs of *A. pelhoensis* (perhaps representing a single clutch) were found in a single mussel. The finding that clutch size varies to this extent among bitterling species has important consequences for understanding oviposition decisions, because spawning season fecundity is similar among bitterling species. Thus, while females of some species oviposit more than 50 clutches, making an identical number of oviposition decisions, others may allocate all their eggs to a single spawning, thereby making only a single oviposition decision. A variable clutch size in bitterling was reported by Kondo et al. (1984) for Japanese species, and up to 200 eggs may be oviposited in one spawning event in *Acheilognathus longipinnis* from Japan (Ogawa et al., 2000). Notably, our results show that the more eggs that were laid, the more general the pattern of host use was, as shown by *Rhodeus fangi* that used a broader range of host species in 2005 when more eggs and embryos were recovered (Table 2). Notwithstanding the above limitations, the long-term experiment clearly showed that the suitability of different mussels as hosts varied among bitterling species and subsequent experiments confirmed those differences.

In the short-term experiment (Experiment 2), direct tests with a subset of species showed that both host preference and host suitability were important in shaping the final pattern of egg and embryo distribution among host species. *Rhodeus ocellatus*, a generalist, used all four hosts offered, although it showed a consistent preference for *Anodonta globosula* (Figs. 1–3). In contrast, the eggs and embryos of *R. sinensis* were found exclusively in *Unio douglasiae* in the long-term experiment (Table 2); both male and female fish strongly preferred *U. douglasiae* and oviposited solely into them (Figs. 2, 3). However, after 24 h exposure *R. sinensis* eggs were also found in the other three mussel species tested (Fig. 1). This result suggests that even in an apparent strict specialist, host use may be affected by the quality of individual hosts, and *R. sinensis* clearly retains an ability to oviposit in several mussel species. Mussel quality declines as more eggs are deposited on their gills. In the short-term experiment, it appeared that as the favoured mussel species (*U. douglasiae*) filled with eggs, and its quality as a spawning site declined, *R. sinensis* switched spawning to the other three mussel species available. Once ovulated eggs must be spawned, otherwise they are aborted by females (M. Reichard, personal observation). Such flexibility in host preference is also shown in the European bitterling, *Rhodeus amarus*, which switches host choice adaptively to minimize density-dependent mortality of embryos in super-parasitized mussels (Smith et al., 2000a, 2000b), and supports our prediction that host preference is modulated in relation to temporal changes in relative host quality.

Notably, in the long-term experiment, we found a clutch of 15 decaying eggs of *R. sinensis* on the gills of *Lamprotula caveata*. We have similarly observed high egg mortalities for *Tanakia tabira* and *T. limbata*, two Japanese bitterling species that have spawned their eggs on the gills of European mussels (C. Smith, unpublished data). These observations suggest that *R. sinensis* may not be able to complete their development in certain hosts, despite using them for oviposition when preferred hosts are unavailable or already filled with eggs. A comparable situation has been observed in the pierid butterfly *Pieris occidentalis* that used *Thlaspi arvense* for oviposition even though its larvae could not complete their development on this host (Chew, 1977). However, further research is needed to investigate maladaptive host use. For example, *U. douglasiae* is typically the most abundant mussel encountered in the field (Liu et al., 2006; M. Reichard, unpublished data) and *R. sinensis* may not resort to using other host species in nature.
The prediction that mussel morphology and physiology determine their suitability for bitterling embryo development and oviposition preference is only partially supported by our data. In the generalist *R. ocellatus*, where this prediction could have been tested, the rank order of host preference matched host total respiration rate, with mussels that consume less oxygen being most preferred. This result is consistent with the host quality hypothesis (Smith *et al.*, 2001, 2004) that predicts adaptive mussel use with respect to host quality for developing embryos that female bitterling can detect (Smith *et al.*, 2004). However, other bitterling species showed a preference for other hosts (Table 2). For example, most eggs and embryos of *Acheilognathus chankaensis* were found in *L. caveata*, a mussel with the highest oxygen consumption (Fig. 5).

Host preference by bitterling might also be influenced by the anatomical structure of the host mussel gill. Four broad groups of mussel gills can be distinguished among the Unionidae based on the gill structure (Wu, 1998). These types vary in their gill complexity, with the simplest gill type being found in the Ableminae and the most complex gills being displayed by the Anodontinae. Eggs and embryos of bitterling display unique adaptations that enable them to survive in a mussel gill chamber (Fukuhara *et al.*, 1982; Suzuki *et al.*, 1986) and bitterling eggs and embryos may be adapted to specific gill types (Liu *et al.*, 2006). For example, *Rhodeus* embryos have two wing-like yolk projections (Aldridge, 1999; Suzuki *et al.*, 1986), which are lacking in *Acheilognathus* spp. embryos. Also, all species of *Rhodeus* and some *Acheilognathus* possess scaly tubercles on their yolk-sac (Fukuhara *et al.*, 1982), which may be suited for a particular host species and may serve in lodging the embryo in the gills of its host. In addition, the reproductive anatomy of adult bitterling, such as ovipositor length, varies widely among species (Kitamura, 2006b) and could be tailored to oviposition in certain mussel species. At present, too few data are available to examine further the effects of these adaptations on host use.

We propose that bitterling oviposition decisions are based on a hierarchy of host traits. At the broadest level of host preference, bitterling (subfamily Acheilognathinae) respond to host suitability that is affected by a general mussel anatomy, including siphon and gill size. At this level, bitterling are able to oviposit into mussels from the families Unionidae and Margaritiferidae (Smith *et al.*, 2004). The anatomy of margaritiferid mussels is similar to the least complex gill structure found in the Ableminae (gill type A); they lack true water tubes or vertical septa and are considered a primitive group related to the Unionidae (Dillon, 2000). In contrast, no bitterling eggs or embryos have ever been reported from mussels belonging to the Corbiculidae, Sphaeriidae or Dreissenidae, although representatives of these families co-exist with bitterling over their entire range.

At an intermediate level of host preference, bitterling appear to show broad adaptation to a particular group of host mussels (Liu *et al.*, 2006). These are mussel species that may all be used for successful embryo development (egg and embryo may reside in the mussel gills and adult fish are adapted to locate a host and oviposit into it), but given a choice bitterling show varying degrees of preference for them. In most bitterling species we tested, the majority of eggs and embryos were found in mussels with a simple gill structure (types A and B). However, *Acheilognathus peihoensis* preferred *Anodonta globosula* with the most complex gill type (type D) and *R. ocellatus* readily used mussels with all gill types. Like *R. ocellatus*, the European bitterling *R. amarus* is a generalist, but prefers *Unio* spp. hosts (type B gills) over *Anodonta* spp. (type D gills) when given a choice (Smith *et al.*, 2004).

Different bitterling populations may vary in their preference for host species (Kondo *et al.*, 1984; Kitamura, 2006b). These preferences could relate to the current state of the co-evolutionary
dynamics between bitterling and mussel (Thompson and Cunningham, 2002; Reichard et al., 2006). Notably, bitterling species with wide geographical distributions in Japan show high variance in ovipositor length and egg size and shape, which may represent adaptations to the particular mussel faunas they use as spawning hosts (J. Katamura, personal communication). At present, we have no quantitative data on how consistent the oviposition decisions are in individual fish, with possible underlying consequences for the existence of gentes (host-specific female races) within bitterling species analogous to those in the European cuckoo (Davies and Brooke, 1989), or speciation by host switching (Sorensen et al., 2003), although this is the subject of ongoing studies.

At the finest level of host preference, bitterling also discriminate among individuals within a host species, with discrimination based on cues that relate to some aspect of host quality (Smith et al., 2000a, 2004). Mussel oxygen consumption positively correlated with mortality rate of European R. amarus eggs and embryos on mussel gills, but negatively with host preferences (Smith et al., 2000a, 2000b, 2001). Mussel ventilation rates also correlated with the host’s ability to eject developing eggs and embryos (Mills and Reynolds, 2002a, 2002b). In the present study, we used two measures of the respiration rate that both combined the effects of oxygen availability and ventilation rate into a single value. Thus, total respiration rate relates to the conditions that bitterling perceive when inspecting a mussel before oviposition and the conditions the embryos experience in the mussel gill chamber. We found that the generalist R. ocellatus preferred hosts with the lowest total respiration rate; the results match those for R. amarus. However, other species showed specific host preferences, with A. chankaensis, for example, preferring hosts with the highest total respiration rate (Table 2). A complication is that total respiration rate varied with gill anatomy. Thus mussels with the simplest gill structure showed the lowest efficiency in oxygen uptake, whereas mussels with the most complex gills had the highest values for total respiration rate. Consequently, the results of the present study do not allow us to differentiate between the effects on host preference of adaptation to gill structure from variation in host quality.

The relative respiration rate (total respiration rate per unit of dry body mass) likely correlated with the velocity of water circulating through the mussel gills, and the ability of a mussel to eject R. ocellatus eggs immediately after spawning. This correlation points towards the velocity of the water circulating in the mussel gill chamber as the possible mechanism behind egg ejections. Mills and Reynolds (2002a, 2000b) found that the ventilation rates of four European mussels matched their ability to eject R. amarus eggs and embryos and our results support their finding using an independent data set.

In summary, we found interspecific differences among bitterling species in their use of mussel hosts. Bitterling species varied in the level of their host specificity and identity of the preferred host. Host preference was flexible even among apparently specialized species and fish switched their preferences adaptively when the quality of individuals of preferred host species declined after receiving many eggs. Mussels varied considerably in their response to oviposition through egg ejections. Host preference by a generalist bitterling species positively correlated with host quality measured as the efficiency of the mussel gills to extract oxygen from inhaled water. Host ability to eject bitterling eggs correlated positively with their relative respiration rate, perhaps due to a higher velocity of the water circulating through the mussel gill chamber. Ongoing research will examine the roles of morphological, physiological, and behavioural adaptations of bitterling to use specific hosts.
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