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The costs and benefits in an unusual symbiosis: experimental evidence that bitterling fish (*Rhodeus sericeus*) are parasites of unionid mussels in Europe

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Abstract

Interspecific symbiotic relationships involve a complex network of interactions, and understanding their outcome requires quantification of the costs and benefits to both partners. We experimentally investigated the costs and benefits in the relationship between European bitterling fish (*Rhodeus sericeus*) and freshwater mussels that are used by *R. sericeus* for oviposition. This relationship has hitherto been thought mutualistic, on the premise that *R. sericeus* use mussels as foster parents of their embryos while mussels use *R. sericeus* as hosts for their larvae. We demonstrate that *R. sericeus* is a parasite of European mussels, because it (i) avoids the cost of infection by mussel larvae and (ii) imposes a direct cost on mussels. Our experiments also indicate a potential coevolutionary arms race between bitterling fishes and their mussel hosts; the outcome of this relationship may differ between Asia, the centre of distribution of bitterling fishes, and Europe where they have recently invaded.

Introduction

A symbiosis is a close ecological relationship between individuals of two (or more) species. The nature of the relationship between symbiotic partners is variable, ranging from mutualism, where both partners obtain a fitness benefit from the relationship, through commensalism where one species benefits whilst the other is unaffected, to a parasitic exploitation of one species by the other (Thompson, 1994; Paracer & Ahmadjian, 2000).

Symbiotic relationships are dynamic; some evolving to stable equilibrium, while others represent rapidly coevolving arms races between species. Further, benefits to one partner are often acquired through costs to the other partner, giving rise to a complex network of interactions (Bronstein, 2001; Holland *et al.*, 2004). For example, a mutualism between the senita cactus (*Pachycercus schottii*) and senita moth (*Upiga virescens*), where adult moths pollinate the cactus while their larvae consume its seeds, appears to be maintained by excess flower production and ensuing fruit abortion that regulates moth population density (Holland *et al.*, 2004). In contrast, the coevolutionary arms race seen between avian brood parasites and their hosts involves a complex system of egg-laying behaviour and egg mimicry by the parasite, and nest concealment, egg discrimination and parasite rejection by the host (Rothstein & Robinson, 1998). In addition, it is now recognized that symbiotic (mutualistic, commensal and parasitic) relationships can be labile, with symbionts varying across their geographic ranges in their degree of co-adaptation. For example, the interaction between the herbaceous plant *Lithophragma parviflorum* and the parasitic moth *Greya politella* is mutualistic in some habitats, but commensal or antagonistic in neighbouring habitats (Thompson & Cunningham, 2002). Thus, across their geographic range symbionts may coevolve towards a complex spatial mosaic of coevolutionary hotspots and coldspots, depending on

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the community context of the interaction and leading to complex patterns of association between symbionts among habitats. However, only by quantifying the costs and benefits to both partners in a symbiosis can an understanding of the nature of such relationships be obtained (Tillberg, 2004).

Important insights into symbioses can be gained from studies that involve species that have recently invaded outside their historical range (Cruz *et al.*, 1998). The brown-headed cowbird (*Molothurus ater*) experienced a dramatic range expansion and population increase following European settlement and deforestation of North America and this brought it in contact with many bird species that were previously unexposed to brood parasites. Recent host species (and also parasite-allopatric populations) either fail to show any response, or show only a weak response to parasitism, due to an 'evolutionary lag': host populations have not yet evolved adaptive responses to their new parasites. As a consequence these recent hosts are especially vulnerable to cowbird exploitation, with severe consequences for population size (e.g. Briskie *et al.*, 1992; Trail & Baptista, 1993; Hosoi & Rothstein, 2000).

Here, we investigate the nature of the symbiosis between the European bitterling (*Rhodeus sericeus*), a small cyprinid fish, and freshwater unionid mussels (*Anodonta* spp. and *Unio* spp., family Unionidae). *Rhodeus sericeus* lay their eggs in the gill chambers of unionid mussels, where they develop into free-swimming larvae (biology reviewed by Smith *et al.*, 2004). The unionid mussels used by *R. sericeus* for oviposition have larval stages (termed glochidia) that are obligate parasites of freshwater fishes. The relationship between *R. sericeus* and unionid mussels involves a two-way interaction, making the system unusually tractable to directly investigate the costs and benefits of their symbiosis. Unionid mussels may eject *R. sericeus* embryos prematurely (before development is complete), while *R. sericeus* make sophisticated oviposition decisions to avoid ejections (Smith *et al.*, 2000; Mills & Reynolds, 2002). Several studies have attempted to identify and quantify the costs and benefits of the symbiosis (Smith *et al.*, 2001; Mills & Reynolds, 2002, 2003), but none have succeeded in finding conclusive evidence of the nature of the relationship (Mills & Reynolds, 2003).

During the spawning season (late April–June in our study area) male *R. sericeus* 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. Between 1 and 6 eggs are released at each spawning, and mussels may harbour over 250 developing embryos on their gills at the peak of the *R. sericeus* breeding season. The eggs of *R. sericeus* are large, elliptical and yolk-rich. *Rhodeus sericeus* embryos develop inside the mussel for 3–6 weeks, eventually leaving the mussel as well-developed larvae (Smith *et al.*, 2004).

Unionid mussels used by *R. sericeus* for oviposition live buried in the fine sediments of rivers and lakes. They expose their inhalant and exhalant siphons to filter suspended food items from the water and to obtain oxygen. Female unionids brood their glochidia larvae in modified portions of their gills, the marsupia. When glochidia are mature, they are expelled into the water column. They are composed of a tiny (0.10–0.45 mm) hinged valve that snaps shut on contact with a fish, typically attaching to their gills (*Unio* spp.) and fins (*Anodonta* spp.). Once attached, the glochidia are encysted by host tissue, remaining attached for several weeks and obtaining nourishment from their host. Attachment to fishes is obligatory for their successful development and is also thought to serve in dispersal. High levels of infection by glochidia may be lethal to fish (Myers & Millemann, 1977). For details on unionid biology and reproduction see Bauer & Wächtler (2000). Four species of unionid mussels commonly co-occur with *R. sericeus* in Europe and all of them are used for oviposition; *Unio pictorum* (L.), *Unio tumidus* (Philipsson), *Anodonta anatina* (L.) and *Anodonta cygnea* (L.). Other unionid (and margaritiferid) species are readily used when available (reviewed in Smith *et al.*, 2004).

The relationship between *R. sericeus* and mussels has popularly been considered mutualistic on the premise that bitterling use mussels as spawning sites, while the mussel benefits by using bitterling as hosts for their glochidia (e.g. Wheeler, 1978). However, recent studies have shown the evidence for a mutualistic relationship to be weak. First, a field study (Kadlec *et al.*, 2003) suggested that *R. sericeus* become infected with considerably fewer glochidia than other fishes, questioning the benefits of the symbiosis for both partners. Second, *R. sericeus* embryos have been reported to have an adverse effect on mussel physiology; possibly competing with their host mussel for oxygen (Smith *et al.*, 2001), though there is no direct evidence that a reduction in oxygen availability translates into a fitness cost to the mussel (Mills & Reynolds, 2003). Given that there is no study on the direct effect of *R. sericeus* embryos on mussels, it is impossible to separate a commensal symbiosis (bitterling benefits but mussels suffer no fitness cost) from a parasitic one. Furthermore, because the evidence of lower infection rates of *R. sericeus* by mussel glochidia has been observational and concerns fish of varying age, it is difficult to exclude the influence of confounding effects such as habitat use, body size or immunological response after previous infections (Smith *et al.*, 2004).

An understanding of a symbiosis is greatly enhanced through the recognition of the evolutionary framework of the relationship (Compton *et al.*, 1996). *Rhodeus sericeus* belongs to the sub-family Acheilognathinae, a group of cyprinid fishes comprising approximately 40 species that all share the same reproductive mode (Arai, 1988). The bitterling fishes are distributed in Eastern Asia, except for *R. sericeus* that is believed to be a recent invader to Central and Western Europe. *Rhodeus sericeus* has a discontinuous distribution across its range. In the east it is reported from the River Amur system, Sakhalin Island and rivers emptying into Peter the Great Bay and Sea of Japan. In the west, it is found in Europe and Asia Minor (Holčík, 1999). Some authors (e.g. Kottelat, 1997) have considered European populations to represent a separate species, *Rhodeus amarus* Bloch.

The occurrence of *R. sericeus* in Central Europe is recent; *R. sericeus* were first recorded in the middle Danube system in the mid-19th century. Prior to this period, *R. sericeus* was present in localized refugia in the Ponto-Caspian region and Transcaucasia (Bogutskaya & Komlev, 2001). Thus, the mussel species used by *R. sericeus* for spawning in the west of its distribution appear to be hosts with a short evolutionary association with bitterling compared with Asian bitterling populations. Consequently, if bitterling and mussels have co-evolved, the response of European mussels to bitterling eggs and embryos may be different from that of Asian mussels that have a longer co-evolutionary association with multiple bitterling species, including *R. sericeus*.

Research on *R. sericeus* biology over the past 10 years has established this fish as a valuable model for studies in behavioural and evolutionary biology (Smith *et al.*, 2004). Here we focus on clarifying the nature of the relationship between *R. sericeus* and its mussel hosts in the European part of its distribution by measuring the costs and benefits associated with their symbiosis. Specifically, we experimentally compared (i) the incidence and level of glochidial infections between *R. sericeus* and four control fish species and (ii) the growth rate of unionid mussels exposed to different densities of *R. sericeus* embryos. We included species of both European and Asian origin as controls in our analyses to account for possible co-evolutionary processes. We established that *R. sericeus* is a parasite of European mussels, but the situation in Asia, at the centre of the distribution of bitterling and unionid mussels, is different.

Materials and methods

Glochidial infection (experiments 1, 2 and 3)

Experiments 1, 2 and 3 addressed the potential benefits to mussels from bitterling of their use as host fish for their larval stages. We conducted two independent experiments to test whether glochidia of European mussels attach to *R. sericeus* and to compare the intensity of infection between *R. sericeus* and the three control fish species, *Perca fluviatilis* L., *Rutilus rutilus* (L.) and *Carassius auratus* (L.). *Perca fluviatilis* (Percidae) and *R. rutilus* (Cyprinidae) are the most common species at our study sites and are widely distributed throughout Europe (Wheeler, 1978). *Carassius auratus* (Cyprinidae) is a recent invader to Central Europe; it became widespread after the 1970s following its incidental release with a stock of Asian cyprinids for aquaculture in 1954 (Holčík, 1978).

In experiment 1 we compared the levels of infection by *A. anatina* glochidia in eight experimental cages situated in Lake Hvězda (area 9.4 Ha) in which *A. anatina* are common. The experiment lasted from 27 October to 18 November 2003 (*A. anatina* begin release of glochidia in late October and complete their release by the end of February). Two individuals of each of the four fish species tested were housed in a net container (33 × 33 × 55 cm; mesh size 3 mm) and placed around the lake 2–10 m from the shoreline (water depth 0.8–1.4 m). Fish were collected from adjacent lakes and a subsample ($n = 3$ *per* species) was dissected to estimate initial intensities of infection by glochidia. Two fish *per* species were used in each replicate as insurance for possible losses of fish over the course of the experiment. Only *P. fluviatilis* were infected (mean 21 ± 3.5 glochidia *per* fish) prior to the experiment. Young-of-the-year (YOY) fish were used to avoid effects of possible immunization from previous infections and to reduce the chance of predation by *P. fluviatilis*, which is a predator of the other test species when older. Experimental fish were of similar size (mean ± SE body size: *P. fluviatilis* 60 ± 1.7 mm; *R. rutilus* 59 ± 1.8 mm; *R. sericeus* 46 ± 1.9 mm; *C. auratus* 38 ± 2.4 mm). Fish had access to natural food (plankton) throughout the experiment. After 3 weeks the cages were recovered, fish were transported to a field station and immediately dissected. The number of glochidia on fish fins, body and gills was recorded.

In experiment 2 the levels of infection by larval *Unio* spp. were tested. Sixteen netted containers (see above) were placed into an experimental pool (12 × 6 m) at the garden of the Institute of Vertebrate Biology, Brno, Czech Republic. Each container contained three *U. tumidus* and four *U. pictorum* mussels, and two individuals of *P. fluviatilis*, *R. rutilus* and *R. sericeus* (mean ± SE body size: 41 ± 0.6 mm, 39 ± 0.6 mm and 37 ± 0.6 mm, respectively). *Carassius auratus* was not used because we were unable to capture sufficient numbers of young *C. auratus* of the appropriate size class. Experiment 2 lasted from 28 July to 31 August 2004 (*Unio* spp. release glochidia from late May to September). Control fish were dissected prior to the start of the experiment ($n = 5$ *per* species) and only *P. fluviatilis* (mean = 1 ± 0.5 SE glochidium per fish) were found to be infected. Other features of the experiment were identical to experiment 1. Glochidia of *U. pictorum* and *U. tumidus* cannot be distinguished readily, and data for infection by both species were pooled for analysis.

The aim of experiment 3 was to compare the susceptibility of *R. sericeus* and Chinese rosy bitterling *Rhodeus ocellatus* Kner (of Asian origin) to the glochidia of the Chinese pond mussel (*Anodonta woodiana* Lea). *Rhodeus* *ocellatus* and *A. woodiana* co-occur and are widespread throughout most of Central and Eastern China, the centre of distribution of the bitterling fishes (Arai, 1988). Two individuals each of *R. sericeus* and *R. ocellatus* and one female *A. woodiana* with ripe glochidia were housed in 70 L aquaria. After 36 h, *A. woodiana* were removed and one individual of each bitterling species dissected and the number of glochidia recorded as described above. After 6 days from the start of the experiment, the remaining two fish were dissected. Ten replicates were completed.

The effect of *R. sericeus* embryos on mussel growth (experiment 4)

We conducted experiment 4 to investigate whether *R. sericeus* embryos causes a reduction in fitness to mussels. Two mussel species were used, *U. pictorum* and *A. woodiana*. *R. sericeus* prefers *U. pictorum* for oviposition; it has the lowest ejection rate among European mussels (*ca*. 20%, with no ejection at spawning; Mills & Reynolds, 2003) and may contain up to 150 *R. sericeus* embryos (typically 40; Reichard *et al*., 2004a). *Anodonta woodiana* inhabits numerous river basins in Eastern Asia (from the River Amur basin in the north to the subtropics) and is a common host of several bitterling species, including *R. sericeus* (Hirai, 1965; Nagata, 1985; Holčík, 1999). The ejection rate of *A. woodiana* is high; over 50% of *R. sericeus* eggs were ejected immediately after oviposition and no embryo survived the entire incubation period (M. Reichard, M. Przybylski, P. Kaniewska, C. Smith, unpublished data) and a marginally lower rate of ejections immediately following spawning was observed for the eggs of *R. ocellatus* in Central China (M. Reichard, H. Liu, C. Smith, unpublished data). *Anodonta woodiana* has been widely introduced outside their home range (Waters, 1997) and inhabits several localities with artificially elevated water temperature in Central Europe. *Anodonta woodiana* used in the experiment were collected from a canal connecting the water-cooling system of the Konin electric power plant with Lake Licheňskie in Poland where they were introduced in the mid 1980s. *Unio pictorum* were collected from Lake Hvězda.

In this experiment, mussels were placed in sand-filled basins (diameter 23 cm, depth 5 cm) around the perimeter of the experimental pool. The pool contained approximately 250 *R. sericeus* that were collected from the River Kyjovka prior to the start of the bitterling spawning season. Fish were fed with frozen chironomid larvae and foraged on algae that established on the walls of the pool. The pool contained sufficient phytoplankton densities to sustain the mussels stocked in the pool.

A total of 12 replicates were used, each comprising a pair of basins containing a single *U. pictorum* and *A. woodiana*. Initially, all mussels were uncovered and exposed to *R. sericeus* spawning. After 24 h one set of mussels in each replicate was enclosed inside a net container (mesh size 5 mm, allowing free access to phytoplankton food) to prevent further spawnings by *R. sericeus*. Consequently, one basin (exposed for 24 h) contained mussels with a low embryo density treatment, and the other (exposed throughout the experiment) with a high embryo density treatment. The experiment lasted from 13 May to 22 September 2004. Mussels were measured for their total length (TL, along the longest axis of their shell) to the nearest 0.01 mm using digital callipers at the beginning of the experiment and re-measured three times (on 10 June, 9 July and 22 September, i.e. after 4, 10 and 19 weeks) to examine immediate, short-term and longer-term effects of *R. sericeus* embryos on mussel growth.

Four weeks after the start of the experiment, three mussels of each species were dissected in each treatment. The number of *R. sericeus* eggs and embryos in the mussel gills was recorded. The period of 4 weeks covered the peak of *R. sericeus* spawning and was shorter than the typical duration of *R. sericeus* embryonic development (Smith *et al*., 2004). This allowed us to estimate the density of *R. sericeus* embryos in mussels before they emerged. At the end of the experiment, all experimental mussels were dissected and *A. woodiana* were checked for glochidia. Since we used no sexually mature female mussels in this experiment, all fish from the experimental pool were released back to their natal river.

The effect of *R. ocellatus* embryos on mussel growth (experiment 5)

Experiment 5 was conducted to investigate the fitness costs to mussels that have a long evolutionary history with bitterling fishes. Only short-term effects were investigated due to time constraints. The experiment was conducted at the Institute of Hydrobiology of the Chinese Academy of Sciences in Wuhan, China and lasted from 27 April to 15 May 2004. A total of 95 *R. ocellatus* (sex ratio: 1 : 1) were housed in a 300 L glass tank with 32 *Anodonta globosula* Lea mussels placed in sand-filled basins. Half the mussels were covered with netting (mesh size 5 mm) to prevent *R. ocellatus* spawning in the mussels, while the others were left exposed. Mussels were fed with phytoplankton cultured in an outdoor pond and fish were fed twice daily with live bloodworm. *Rhodeus ocellatus* and *A. globosula* were obtained from lakes in Hubei province, China, where both species are common. *Anodonta globosula* were chosen because they are fast growing and are known to be hosts of several bitterling species, including *R. ocellatus* (M. Reichard, H. Liu, C. Smith, unpublished data). Other aspects of the experiment were similar to experiment 4.

Data analyses

The epidemiological characteristics of glochidial infection in fish were estimated according to Bush *et al.* (1997).

The mean abundance is the average number of glochidia among all fish examined of a given species (including uninfected individuals). Prevalence is the proportion of infected fish in the sample. ANCOVA (with fish length as a covariate) was used to compare the number of glochidia *per* individual fish among the fish species tested. Some experimental fish in experiment 1 escaped from net containers. Consequently all those fish recovered at the end of experiment 1 were dissected and used to calculate epidemiological characteristics. The mean number of glochidia *per* fish of each species in a replicate was used for analysis if both individuals were recovered to avoid pseudoreplication. Note that concordant results of epidemiological characteristics were obtained when this smaller sample was used. The relatively low levels of glochidial infection compared to studies that have recorded mortality related to glochidial infection (Myers & Millemann, 1977) do not indicate glochidia-related mortality. In experiment 2, only one individual of each species was dissected because the sample size was larger and recovery rate was almost 100%.

The growth rate of mussels in the high and low *R. sericeus* embryo treatments were compared using ANCOVA, with initial mussel size as the covariate. Mean daily increments were calculated for every individual by dividing the total growth increment for the period between two measurements by the number of days; allowing growth rates to be compared over the entire course of the study. Means are presented with 1 SE (±SE).

For all experiments, we used the numbers of fish and mussels that we considered sufficient to detect statistically significant effects, based on our pilot studies and observational evidence of glochidial load from previous studies. Because the number of replicates in the mussel growth experiments might be considered relatively low, we estimated the effect size measure (Cohen's *d*) in addition to the conventional significance testing. Cohen's *d* measures the magnitude of a treatment effect as the standardized difference between two means by comparing the overlap in the distribution between the two data sets independently of sample size. Cohen's *d* was calculated as the difference between the means divided by pooled standard deviation (the square root of the average of the squared SD) (Cohen, 1988).

Results

Glochidial infection

There were significant differences in the infection rate by glochidia of *A. anatina* among the four fish species (ANCOVA on $\log_{10}$ transformed data, $F_{3,22} = 14.7$, $P < 0.001$). Three different groups were detected by Tukey *post hoc* tests on the glochidia abundance data (Fig. 1). The highest abundance of glochidia was recorded on *P. fluviatilis* (mean abundance: 48.4; range: 19–107; prevalence: 100%; $n = 14$), followed by *R. rutilus* (mean abundance: 12.1; range: 3–37; prevalence: 100%; $n = 11$) and *C. auratus* (mean abundance: 9.7; range: 2–32; prevalence: 100%; $n = 6$) and the lowest on *R. sericeus* (mean abundance: 1.4; range: 0–10; prevalence: 40%; $n = 10$). The same results were obtained when the infection rates of *P. fluviatilis* were reduced by 21 glochidia *per* fish (the mean infection rate of control *P. fluviatilis* dissected before the start of the experiment). No effect of fish body size on the infection rate was detected (ANCOVA on $\log_{10}$ transformed data, $F_{1,22} = 0.5$, $P = 0.49$).

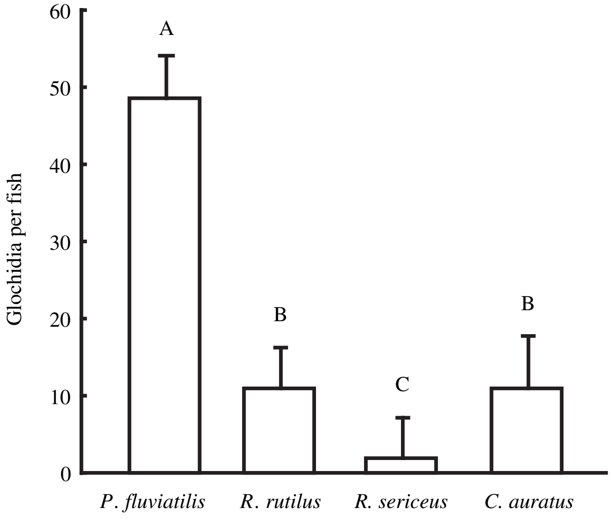

Fig. 1 Mean (+1 SE) abundance of *Anodonta* spp. glochidia among four fish species in experiment 1. The same letters denote groups that did not statistically differ (Tukey *post hoc* tests on $\log_{10}$ transformed data).

In experiment 2 with *Unio* spp., only *P. fluviatilis* (mean abundance: 10.4; range: 0–51; prevalence: 38%) was found to be infected. No glochidia were found on *R. rutilus* or *R. sericeus*. A high level of protozoan infections was recorded on the gills of most *R. rutilus*, *R. sericeus* and uninfected *P. fluviatilis*, which may have resulted in lower infection rate by glochidia, possibly through competitive interactions.

In experiment 3 with *A. woodiana*, *R. sericeus* had a higher initial infection rate (mean abundance: 10.7; range: 2–32; prevalence: 100%) than *R. ocellatus* (mean abundance: 2.5; range: 0–16; prevalence: 50%) and this difference was significant (Wilcoxon paired test: $Z_9 = 2.8$, $P = 0.005$). Six days after exposure *R. sericeus* infections were low (mean abundance: 0.3; range: 0–2; prevalence: 20%) and no glochidia were found on *R. ocellatus*. All three glochidia found on the fins of *R. sericeus* were not encysted into the host tissue and were unlikely to be able to complete their development.

The effect of bitterling embryos on mussel growth

Mussel dissections 4 weeks after the start of experiment 4 confirmed that the group of *U. pictorum* continuously exposed to bitterling spawning received considerably more bitterling embryos (mean: 38.3 ± 9.8 SE, range: 22–56; embryos, $n = 3$) compared with the group of *U. pictorum* exposed for only 24 h (3.3 ± 2.0 SE, range: 0–7 embryos, $n = 3$). This difference was significant (ANOVA, $F_{1,4} = 12.1$, $P = 0.025$, $d = 2.845$). In contrast, only a single *A. woodiana* contained bitterling embryos (mean 1.0 ± 1.0 SE, range: 0–3 embryos) at the high-density treatment and none at the low-density treatment (Fig. 2).

The growth rate of *U. pictorum* in the high embryo density treatment was significantly reduced in comparison with the low-density treatment. This effect was apparent after 4 weeks (ANCOVA, $F_{1,20} = 6.2$, $P = 0.022$, $d = 1.102$), and was even stronger after 10 weeks (ANCOVA, $F_{1,14} = 9.5$, $P = 0.001$, $d = 1.582$) and still significant after 19 weeks (ANCOVA, $F_{1,14} = 6.1$, $P = 0.027$, $d = 0.886$) (Fig. 3a). In contrast, there was no difference in the shell growth between the two experimental groups of *A. woodiana* over the study period (ANCOVA, $F_{1,21} = 1.6$, $P = 0.22$, $d = 0.526$ after 4 weeks; $F_{1,15} = 0.003$, $P = 0.96$, $d = 0.068$ after 10 weeks; $F_{1,15} = 0.7$, $P = 0.80$, $d = 0.128$ after 19 weeks) (Fig. 3b). The growth rates of *A. woodiana* were highest in May and June and declined later in the season. The growth rates of *U. pictorum* remained similar throughout the study period (Fig. 3). Mean overall growth increments were 1.12 ± 0.27 mm at the high embryo density treatment and 2.11 ± 0.49 mm at the low-density treatment in *U. pictorum*, and 3.83 ± 0.28 mm in the high-density treatment and 3.69 ± 0.43 mm in the low-density treatment in *A. woodiana*.

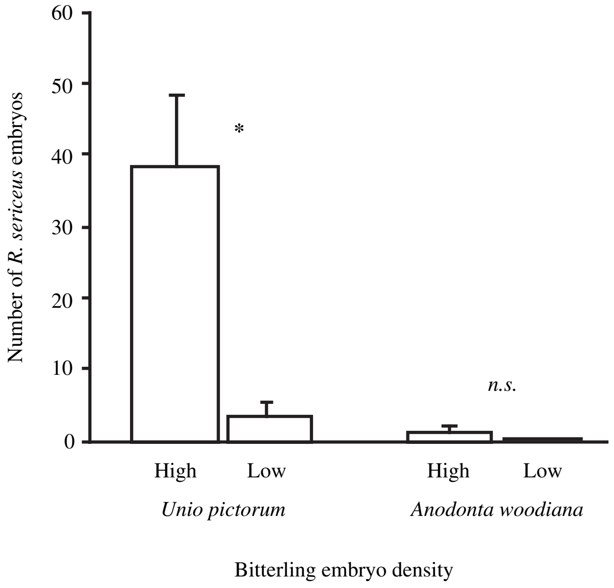

Fig. 2 Number of *R. sericeus* embryos on host mussel gills after 4 weeks in experiment 3. Mussels at the high-density treatment were exposed to *R. sericeus* spawning for 28 days, mussels at the low-density treatment for 24 h. The asterisk denotes a significant difference at the 0.05 level.

In the growth experiment with *A. globosula* and *R. ocellatus*, a significant increase in shell growth was detected in mussels in the low embryo density treatment (mean 0.283 ± 0.108 mm, $n = 16$), though no growth was detected in the high embryo density treatment (mean: −0.041 ± 0.090 mm, $n = 16$) after 19 days. This difference was significant (ANCOVA, $F_{1,21} = 5.2$, $P = 0.031$, $d = 0.816$).

Discussion

We experimentally investigated the cost and benefits to unionid mussels from their symbiosis with European bitterling (*R. sericeus*), a cyprinid fish that lays its eggs in the gill chambers of mussels. Their relationship has been considered mutualistic on the premise that *R. sericeus* use mussels as sites for oviposition and development of their embryos, while mussels benefit by using *R. sericeus* as hosts for their larvae. However, we showed that (i) *R. sericeus* do not serve as hosts of the parasitic larval stages of mussels (Fig. 1), questioning the benefit of the association for the mussel and (ii) the presence of developing embryos on the gills of mussels significantly reduced mussel growth rate (Fig. 3), implying a potential fitness cost to mussels of the association.

Although *R. sericeus* do not host the glochidia of mussels, they may confer fitness benefits to adult mussels. Male *R. sericeus* release sperm clouds over the inhalant siphon of mussels and, given their promiscuous mating system, sperm expenditure over the course of a spawning season is high (Smith *et al.*, 2003). A mean of two sperm releases per minute. has been recorded at the peak of the spawning season over a single mussel (Reichard *et al.*, 2004a). Thus, adult mussels may benefit from ingestion of *R. sericeus* sperm. However, Mills & Reynolds (2003) found no evidence of an effect on mussel growth among those exposed to higher sperm numbers than a control. Thus, mussels are either unable to ingest *R. sericeus* sperm or the benefits of doing so are minimal (Mills & Reynolds, 2003). The lack of any benefits to mussels from the symbiosis implies it is not mutualistic.

To distinguish between a commensal and a parasitic relationship, the costs to the mussel of hosting bitterling embryos must be considered. Smith *et al.* (2001) showed that the difference between oxygen levels in water entering and leaving the mussel gills increased with the number of *R. sericeus* embryos inside them, suggesting that *R. sericeus* embryos compete with their host mussels for oxygen. However, whether a reduction in oxygen level translates into a direct fitness cost is unclear (Mills & Reynolds, 2003; Smith *et al.*, 2004). In the present study, we found experimental evidence for a direct cost to mussels associated with hosting *R. sericeus* embryos.

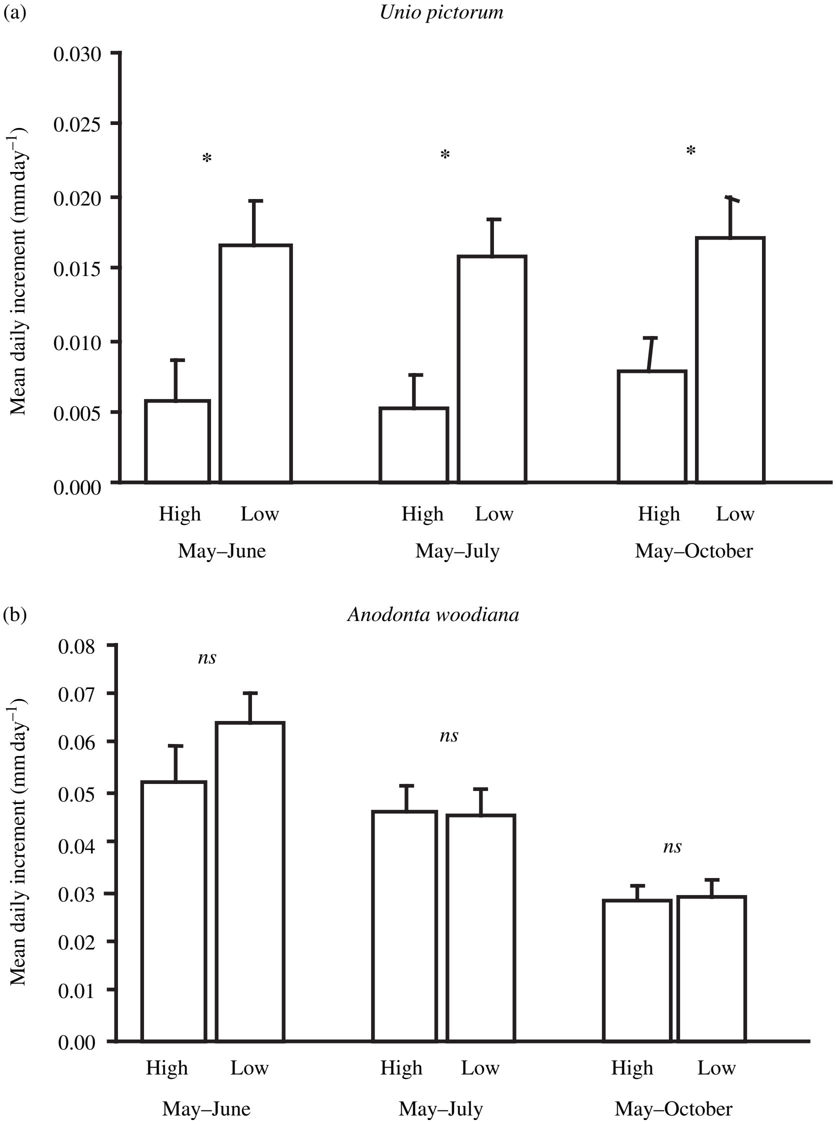

Fig. 3 Mean (+1 SE) daily shell growth of (a) *U. pictorum* and (b) *A. woodiana* mussels at high and low embryo density treatments calculated for the periods May to June (28 days), May to July (70 days) and May to October (131 days) 2004. Asterisks denote a significant difference at the 0.05 level.

Mussels that hosted *R. sericeus* embryos suffered significantly reduced growth over the entire growing season (Fig. 3). Because there is strong evidence across several unionid mussel species (including our study species) that mussel size is positively correlated with fecundity (Bauer, 1994), any reduction in mussel growth will directly translate into a fitness cost. This result demonstrates that *R. sericeus* are parasites of their mussel hosts, at least in Europe.

Both bitterling and their mussel hosts show a range of adaptations to their symbiosis, suggesting that they may have coevolved. Bitterling females develop long ovipositors to place their eggs deep inside the mussel gill cavity; and male sperm has unique adaptations to function in the mussel gill cavity (Guan & Afzelius, 1991; Reichard *et al.*, 2004b). The most striking adaptations are found in the eggs and embryos. These adaptations function either by ensuring the embryo remains lodged in the gill chamber (scaly tubercles on the yolk-sac, wing-like yolk-sac), ensuring an adequate oxygen supply (rapid time to hatching, extensive embryonic circulation, tolerance of low oxygen levels), or ability to reside in the mussel for a protracted period (large, energy-rich yolk-sac) (Kryzhanovsky, 1949). Mussel adaptations to the symbiosis include the premature ejection of bitterling eggs and embryos (Smith *et al.*, 2000; Mills & Reynolds, 2002). To dislodge developing bitterling eggs or embryos, mussels rapidly contract their valves and expel a stream of water. This behaviour may occur either immediately after spawning or later, but typically within the first week of incubation (Mills & Reynolds, 2002; Kitamura, 2005), before the cost of hosting bitterling embryos increases substantially (Smith *et al.*, 2001).

Rhodeus sericeus, as a recent invader of Central and West Europe, may represent a parasite exploiting host populations that did not have sufficient time to evolve counter adaptations. *Rhodeus sericeus* embryos decreased the growth rate of European *U. pictorum*, but not Asian *A. woodiana*. In contrast to *U. pictorum*, which accepts large numbers of *R. sericeus* embryos, *A. woodiana* ejects *R. sericeus* embryos at a high rate. Although direct observation shows that *R. sericeus* readily uses *A. woodiana* for oviposition (M. Reichard, M. Przybylski, P. Kaniewska, C. Smith, unpublished data), most embryos are quickly ejected (Fig. 2). Consequently, we found no decrease in the growth rate of *A. woodiana* mussels exposed to *R. sericeus* spawning (Fig. 3b). This result suggests that the relationship between Asian bitterling species and their mussel hosts may be strikingly different from that between *R. sericeus* and European mussels.

In particular, Asian mussels may have evolved mechanisms to reduce the load of bitterling eggs and embryos they are exposed to, below a rate that suppresses their growth rate. Under this scenario, the bitterling-mussel system may have reached an evolutionary equilibrium, and in Asia their relationship may carry lower costs for mussels or be commensal. However, we found that the presence of the embryos of Asian *R. ocellatus* significantly reduced the growth rate of *A. globosula*, and that *R. ocellatus* avoid glochidia of Asian *A. woodiana*. Currently, there are insufficient data on the cost-benefit relationship for the bitterling-mussel relationship in Asia, though two other studies show *R. ocellatus* to be relatively rare hosts of glochidia (Dudgeon & Morton, 1984; Fukuhara *et al.*, 1986). This indicates that even mussels with a long coevolutionary association with bitterling may be parasitized by bitterling, in spite of their ability to eject bitterling embryos.

Our study demonstrates *R. sericeus* to be a parasite of European unionid mussels. *Rhodeus sericeus* benefits from using mussels as a spawning site, but *R. sericeus* embryos in the gill chambers of mussels reduce mussel growth rate and hence fecundity. Mussels obtain no benefit from their close association with *R. sericeus*, which are able to avoid infection by mussel larvae. However, our results also suggest that the relationship between *R. sericeus* and European mussels may differ from that between Asian bitterling species and their mussel hosts, suggesting a complex network of symbiotic relationships between bitterling and mussels across their range.

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