# SUPPLEMENTARY MATERIAL AND METHODS

**Experimental Animals:** Mussels and fish used in behavioural experiments were collected in the River Kyjovka (Danube Basin, Czech Republic; N 48°46'44'', E 17°01'00'') prior to the onset of the bitterling reproductive season, (6 April 2009) and transported to the facilities of the Institute of Vertebrate Biology (IVB) in Brno, Czech Republic. Native mussels used in the experiments were all *Anodonta anatina*. This species was chosen because it is the dominant native mussel in the River Kyjovka [1] and is readily used by *R. amarus* [2]. Generation time of *R. amarus* is one year; generation time of unionid mussels is between 2-5 years [3]. *Rhodeus amarus* (age 0+) used in the glochidia infestation experiment were collected in the River Štítarský (N 50°16'13''; E 15°11'15''), which has a low density of mussels. All *R. amarus* has been collected prior to the first experiment and held in the lab without any contact with mussels. Therefore, all individuals of *R. amarus* (those used in experiment with *A. woodiana* and those used in experiment with *A. anatina*) have been exposed to any possible natural glochidia infection in the wild for the same time interval and at the same intensity. Control fishes (age 1+) were obtained from a commercial hatchery. *A. woodiana* used as a source of glochidia were collected from the River Kyjovka and *A. anatina* from River Košínský (N 49°27'13"; E 14°39'26").

**Experiment 1- Reproductive success of *R. amarus* populations across varying A. woodiana : *A. anatina* ratios:** A total of 30 experimental populations of *R. amarus* were established in large fiberglass pools (130 x 130 cm) located in the grounds of the Institute of Vertebrate Biology. Each pool was filled to a depth of 60 cm with tap water and furnished with a gravel substrate, artificial plants as refuges, and four sand-filled plastic pots. Each pot contained a mussel; pots kept mussels in fixed positions, but permitted them to adopt natural postures and to filter normally. The ratio of *A. woodiana*: *A. anatina* were 0:4, 1:3, 2:2, 3:1, 4:0, with 6 replicates of each treatment. Mean shell length (±SD) of mussels was 127 ± 8 mm for *A. anatina* and 127 ± 12 mm for *A. woodiana*. Each bitterling population consisted of five males and six females. To standardize female fecundity among populations, fish were sorted into three size groups and each population received two females from each size group. Experimental fish foraged on natural food (algae, detritus, invertebrates) that established in experimental pools and were additionally fed daily with a mixture of frozen chironomid larvae and *Cyclops* nauplii, with an equal amount provided to each population. Several mortalities occurred during the experiment. When a fish died, it was immediately replaced by a

fish of the same size from a stock held in identical pools. In 8 cases we recorded mussel mortalities; in 3 cases mortalities occurred in June, a period with a high rate of *R. amarus* spawning. Because replacement of a mussel would interfere with the estimates of population reproductive success, they were not substituted, but mussel mortality was included in the data analysis. No effect of mussel mortality was observed and results were robust for inclusion/exclusion of mussel mortality in statistical models (see further). For details on the temporal distribution of mussel mortality, see associated data file stored in the Dryad database (doi:10.5061/dryad.q31477f6).

The experiment was conducted from 6 April to 29 September 2009. Populations were monitored daily between 08:00 and 11:00 and any juveniles that had emerged from mussels were collected with a hand net. Assignment of emerged juveniles to specific individual mussels was not possible. Detection of juvenile bitterling following emergence is easy as they shoal at the water surface. Adult fish were removed from pools after complete cessation of reproductive activity (30 July). The first juvenile fish emerged on 24 April; 80% of juveniles emerged before 28 July, 95% before 13 August.

**Experiment 2-Behavioural response of *R. amarus* to *A. woodiana* and native mussels:** We conducted behavioural tests of *R. amarus* host mussel choice at the peak of the reproductive period (from 27 May to 2 June). Tests were conducted in glass aquaria (24 L) with a layer of sand and two plastic sand-filled pots with a single *A. anatina* and *A. woodiana* in each. Pilot studies showed a strong preference for *A. anatina* over *A. woodiana*. Since our principal aim was to test whether *R. amarus* would oviposit in *A. woodiana*, we decreased the quality of *A. anatina* as sites for oviposition by using mussels with a large number of bitterling embryos already in their gills (mean ± SD 50 ± 35 embryos). Bitterling are able to discriminate between mussels that already contain eggs and embryos and prefer mussels that are free of them. This preference is adaptive because egg and embryo survival is density dependent [2]. Mussels were placed in the aquarium and covered with a transparent perforated plastic cup to prevent oviposition but allowing the fish to see and smell the mussels. A male and female *R. amarus* in breeding condition (nuptial colouration in males, extended ovipositor in females) were gently released into the aquarium. Once the male began courtship behaviour (typically within 10 min after introduction) the cup was carefully removed from the mussel and behaviour recording began. Reproductive behaviour directed at specific mussels was recorded for 20 min. or until oviposition. In addition to oviposition, we recorded male and female inspection of mussel siphons (indicating interest in a mussel), male leading behaviour towards a mussel, and sperm release into the mussel inhalant siphon (indicating

male preference for a particular mussel), and female skimming (contact between ovipositor and mussel exhalant siphon, but no oviposition). For details of reproductive behaviours see [4].

After completion of a replicate, fish and mussels were replaced; each individual was used only once. A total of 16 replicates were completed (9 ovipositions). The number of bitterling embryos in mussels was estimated visually prior to the experiment using a mussel opening device, and mussels were dissected and the total number of bitterling embryos were counted after completion of each trial. Newly laid eggs were readily distinguishable from older embryos by their developmental stage. Finally, we investigated whether *R. amarus* chose to oviposit into *A. woodiana* in the absence of an alternative host. The experimental set-up and protocol was identical to choice experiment, except that a single mussel (*A. woodiana*) was placed in the centre of the aquarium and fish behaviour recorded for 40 min.

**Experiment 3-Capability of glochidia to develop on *R. amarus* and control species:** Glochidia compatibility experiments were conducted in September 2010 (*A. woodiana*) and January 2011 (*A. anatina*), corresponding with the peak reproductive season of each mussel species. Gravid mussels were identified in the field by gently opening their valves; swollen demibranchs with ripe glochidia indicated gravidity, and transported to the laboratory. Glochidia were obtained by spontaneous release from mussels and flushing the marsupium with water using a syringe. The viability of glochidia (a subset of 30 individuals for each mussel) was verified by evaluation of their snapping action in a sodium chloride solution [5]. Glochidia from six gravid females of each species with a viability exceeding 90% were pooled and used for inoculation. Experimental fish were infected in an aerated glochidia suspension (volume 0.5 l per fish; all fish infected simultaneously) of dechlorinated tap water containing a mean ± SD of 4570 ± 1279 and 4220 ± 1866 viable glochidia per litre for *A. woodiana* and *A. anatina*, respectively (n = 10 samples of glochidia suspension for each host species). After 15 min of inoculation fish were transferred into water without glochidia for 30 min. to rinse non-attached glochidia. Fish were subsequently placed individually into continuously aerated 5 l plastic tanks, with the bottom covered with a net (mesh size 3 mm) and monitored for glochidia development until the end of the glochidial parasitic phase (30 days, see below). Fish were fed daily with commercial flake fish food. Water temperature was 23.3 ± 0.7 and 21.1 ± 0.4°C during the experiment with *A. woodiana* and *A. anatina* respectively. The difference in temperature reflected seasonal changes in water temperature when the peak of glochidia release is observed for each species. Water was partially exchanged (80% of water

volume) and examined for the presence of glochidia and juvenile mussels at two-day intervals by siphoning the tank. Glochidia and juvenile mussels were collected from siphoned water using filters (mesh size 139 and 507 μm) and identified under a microscope at 10-40x magnification. Glochidia were scored as living juveniles if foot activity or valve movement was observed. These methods enabled us to estimate both the absolute number of juvenile mussels recovered from individual fish and the successful development of initially attached glochidia. Glochidia attach to fins and gills of the fish host and complete development within 2-3 weeks under the ambient temperatures we used [3]. A total of 81 fish were monitored, 5-12 individuals for each mussel (*A. woodiana*, *A. anatina*) and fish (*R. amarus*, *B. barbus*, *L. cephalus*, *P. parva*, *C. gibelio*) combination. One fish (*C. gibelio*) died before the end of the glochidia maturation period and was excluded from analysis. Four control species were used to control for their geographical origin (two European, two East Asian, but introduced to Europe during the 20th century). The use of two control species per each geographic region (each belonging to a different subfamily) aimed at minimizing any phylogenetic effect and made contrast more robust.

**Data analyses:** Reproductive success was compared using Generalized Linear Models (Poisson distribution), with mussel ratio as treatment group and number of juvenile *R. amarus* as the response variable. Given that data were overdispersed (parameter theta >>1), we used a quasi-Poisson error structure in the final model. Pairwise differences between the treatment groups were tested by overlap of their confidence intervals. As some females died during the experiment, we included actual number of females that survived until the end of the oviposition season (end of June) and the end of the experiment (August) as covariates in the initial analysis. Covariates did not significantly improve the model (log-likelihood test based on Maximum Likelihood estimates, $P > 0.65$) and were deleted from final models [6]. Results are presented with the original assignment of populations into treatments. However, we also checked whether mussel mortalities affected the outcome of the analysis. We re-categorized populations to represent the actual number of mussels that survived until >95% of juvenile *R. amarus* emerged (rather than the initial number of mussels); this analysis shifted three populations into a different treatment and yielded outcomes even stronger than those reported for the original assignment ($F_{4,25} = 11.50$, $P < 0.001$). Oviposition data from the behavioural experiment were tested using an exact binomial test. Generalized Linear Mixed Models (GLMM using a Poisson distribution, implemented with the *lme4* package in R) were used to test differences in behavioural interactions with *A. woodiana* and *A. anatina*. Treatments were compared by calculating 95% confidence intervals and their overlap.

Relative development success (the proportion of successfully metamorphosed juvenile mussels in relation to the number of glochidia originally attached) was compared using Generalized Linear Models with a quasi-binomial error structure. Differences in relative development success between five host species were tested by Kruskall-Wallis tests (for *A. woodiana* and *A. anatina* separately), followed by post-hoc multiple comparison testing for pairwise differences.

**References**


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