

The importance of species interactions in conservation: the endangered European bitterling *Rhodeus sericeus* and its freshwater mussel hosts

Suzanne C. Mills and John D. Reynolds

Centre for Ecology, Evolution and Conservation, School of Biological Sciences, University of East Anglia, Norwich, NR4 7TJ, UK

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Abstract

Species conservation may be complicated in symbiotic interactions. Sub-lethal impacts of human activities on one species may be lethal to other species on which they depend. We investigated the impact of two aspects of eutrophication – changes in phytoplankton and oxygen levels – on the interaction between a freshwater fish that is endangered in several countries, the European bitterling, *Rhodeus sericeus*, and the unionid mussel hosts that they require for spawning. Reduced concentrations of algae and oxygen each led to a greater proportion of bitterling embryos being ejected prematurely from the mussel hosts. Reduced algal concentrations also led to reductions in mussel ventilation rates and to mussels spending less time with their valves open. Bitterling mortality may have been due to three causes: direct effects of environmental conditions on embryonic survival, active expulsion by mussels when stressed and through responses by mussels that created internal conditions that bitterling embryos could not survive. Our study shows that the sub-lethal effects of pollution on one species can have lethal effects on another species with which it interacts. Such interactions need to be considered in conservation programmes.

INTRODUCTION

Direct impacts of contaminants on the health and survival of aquatic organisms are well known (e.g. Mason, 1996a). There are also examples of indirect impacts on species through, for example, effects on their predators, prey or parasites (Lafferty & Kuris, 1999). Therefore, the restoration of habitats solely on the basis of lethal toxicity tests of contaminants may not be the most effective conservation method for either the targeted species or for interacting species.

Pollution is recognised as one of the most significant factors causing major declines in populations of freshwater species in many parts of the world (Clark, 1992; Moyle & Leidy, 1992; Winfield, 1992; Lawton & May, 1995; Maitland, 1995; Mason, 1996a). Eutrophication comprises the biological effects of an increase in plant nutrients on aquatic systems and is a widespread and growing problem in lakes, rivers, estuaries and coastal oceans (Clark, 1992; Harper, 1992; Mason, 1996a; Smith, 1998). Most of the excess nutrient input to water bodies is caused by runoff from agricultural lands and as domestic and industrial waste (Mason, 1996a; Carpenter *et al.*, 1998). Eutrophication affects freshwater species either by the deleterious effects of oxygen depletion from the

degradation of organic material or by monospecific algal blooms (Moss, 1988; Freedman, 1995).

The reproductive success of a freshwater fish that is endangered over much of its range, the European bitterling, *Rhodeus sericeus*, depends upon the unionid mussel hosts in which the fish spawn (Reynolds, Debus & Aldridge, 1997; Smith *et al.*, 2000; Mills & Reynolds, 2002b, 2003b). Male bitterling guard a territory containing at least one mussel and court females towards the mussels (Mills & Reynolds, 2003a). Females are attracted to mussels by male bitterling (Candolin & Reynolds, 2001, 2002) and once females have selected a mussel, based on its ventilation rate (Mills & Reynolds, 2002a), they use their long ovipositors to lay 2–4 eggs into water tubes within the gills of a mussel at each spawning. Males release sperm over the inhalant siphon, which is then carried in the mussel's inhalant water current to the eggs, where fertilisation takes place. The embryos are incubated within the mussel's gills for 3–6 weeks and once they have absorbed their yolk sac the larvae leave the mussel via the exhalant siphon (Reynolds *et al.*, 1997). Bitterling cannot reproduce without mussels. Although the mussels' own larvae (glochidia) parasitise the gills or skin of fish, species other than bitterling are the principle hosts, since bitterling appear to have a degree of immunity to them (Aldridge, 1997; Mills & Reynolds, 2003b; and pers. obs.).

European bitterling have been classified as rare and vulnerable over much of the western part of their range, and have disappeared completely from the most polluted areas (e.g. Rhine–Main basin: Lelek, 1980). Widespread

All correspondence to: S. C. Mills. Current address: Department of Biological and Environmental Science, P.O. Box 35 (YAC 4), FIN-40014, University of Jyväskylä, Finland. Tel: +358 - 14 - 260 4199; Fax: +358 - 14 - 260 2321; E-mail: mills@soozie.co.uk

concern has resulted in *R. sericeus* being listed as a protected species in Appendix III of the Bern Convention with legislation in five countries (Maitland, 1994). The IUCN placed *R. sericeus* as vulnerable in France (IUCN, 1995), endangered in Slovenia (Povž, 1992) and it is one of six of the most endangered species in Belgium and protected by law (Bervoets, Coeck & Verheyen, 1990). While the bitterling's unionid hosts have often undergone local declines they are not protected by the Bern Convention, although some species are protected under national legislation (Wells & Chatfield, 1992). Eutrophication has caused the decline of *Anodonta cygnea* and *Unio pictorum* in Poland, where they are Red List classified as endangered and vulnerable, respectively (Dyduch-Falniowska, 1992). In Germany, *R. sericeus* and its four mussel hosts, *A. anatina*, *A. cygnea*, *U. pictorum* and *U. tumidus* are all considered to be highly vulnerable (Blab *et al.*, 1984; Schmidt, 1990). Furthermore, the Red Data List in Switzerland places *A. anatina*, *U. pictorum* and *U. tumidus* as vulnerable while *R. sericeus* is classified as 'strongly endangered' under Swiss legislation (Duelli, 1994; Kirchhofer & Hefti, 1996). In the European areas where mussels survive and bitterling have disappeared it is not known at which life stage these fish are most sensitive to industrial pollution (Lelek, 1980).

In this study we investigated the effect of two typical aspects of eutrophication on the reproductive success of the bitterling. Nutrients such as phosphates, which cause eutrophication, have negative impacts on mussel ventilation and increase bitterling embryo mortality (Reynolds & Guillaume, 1998). These nutrients stimulate the growth of green algae (*Chlorophyta*) and drastically reduce the dissolved oxygen concentration of many systems (Mason, 1996b; Diaz, 2001). Eutrophic lakes and rivers may also have low algal concentrations, as well as low oxygen, if high densities of the herbivorous *Daphnia magna* are present, since these graze on green algae (Borgmann, Millard & Charlton, 1988; Theiss, Zielinski & Lang, 1990; Carvalho, 1994; Carpenter *et al.*, 1998).

Our study focused on the effects of different combinations of concentrations of phytoplankton and dissolved oxygen on the survival of bitterling embryos in mussel hosts. Bitterling show no parental care and their offspring's survival depends on direct impacts of the environment on embryos, as well as the mussels' reaction to changes in environmental conditions. Our aim was to use the bitterling-mussel system as a case study to investigate the consequences of symbioses for conservation, by investigating the direct effects of eutrophication and the indirect effects, through the reactions of their mussel hosts, on the survival of bitterling embryos.

METHODS

Study species

A total of 150 individuals of the mussel species *Anodonta anatina* and bitterling of both sexes were collected from Reach Lode, a tributary of the River Cam,

Cambridgeshire, in southern England, at the point of confluence with Wicken Lode, National Grid Reference: TL 545 696, during March and April 2000. The mussels were collected by hand and the fish were collected using high frequency (600 Hz) pulsed DC Electracatch WFC 12 electrofishing equipment. The mussels were maintained in outdoor pools and fed daily 3 l of a live algal suspension derived from an outdoor pool that had been seeded with *Chlorella vulgaris*. The bitterling were kept in stock aquaria that were aerated continuously with a TETRAtec[®] IN 1000 internal aquarium filter and illuminated by an Aqua-Glow 40W fluorescent aquarium lamp on a 16 h light:8 h dark photoperiod. The fish were fed a mixed diet of live *Daphnia pulex*, *Chaoborus* pupae, *Culex* and *Chironomid* larvae, frozen *D. pulex*, *Tubifex*, *Artemia salina*, dried protein mix and trout pellets. The experiments were carried out in an indoor aquarium facility where the water temperature was maintained between 18–20 °C.

Effect of treatments on bitterling survival

Four mussels were placed in sand-filled round glass containers (10 × 6 cm) and arranged in a square formation within a 300-l aquarium. One male and one female bitterling in reproductive condition were added to the aquarium. The fish were observed until they had spawned twice in each mussel. A mussel was removed after receiving two spawnings, so that the bitterling were forced to spawn in a new mussel, until all four mussels had received two spawnings. We performed 24 replicates of this experiment. The mussels were transferred to a 300-l aquarium that had been separated into four sealed quarters and one mussel was placed in each compartment. After 12 h of acclimation at baseline oxygen and algal conditions (see below), the oxygen and algae concentrations were changed gradually. Four treatments were distributed randomly within each of the four compartments, representing each combination of baseline and 'reduced' oxygen and algae (details below).

Mussels were observed twice daily for 6 weeks and at each observation the numbers of embryos ejected were noted.

Oxygen conditions

The baseline dissolved oxygen concentration used in our experiments was 14 mg l⁻¹. This was based on the mean oxygen concentration for March–May measured from 1993–1998 by the UK Environment Agency at a site 2.1 km upstream from our study site (Reach Lode, Hallards Fen Rd, Cambridgeshire, UK). While rivers that have been less impacted by humans may have higher oxygen concentrations, this level was still adequate to provide a good contrast with the 'reduced' oxygen level typical of more eutrophic habitats. Furthermore, the fact that bitterling are the second most common fish in our study site (after roach, *Rutilus rutilus*) suggests that bitterling populations can thrive under this concentration.

Table 1. Concentrations of chlorophyll *a* ($\mu\text{g l}^{-1}$) and dissolved oxygen (mg l^{-1}) in the four treatments in the two experiments

Treatment	Effects on bitterling experiment		Effects on mussels experiment	
	Chl <i>a</i> ($\mu\text{g l}^{-1}$) $\bar{X} \pm \text{SE}$	O ₂ (mg l^{-1}) $\bar{X} \pm \text{SE}$	Chl <i>a</i> ($\mu\text{g l}^{-1}$) $\bar{X} \pm \text{SE}$	O ₂ (mg l^{-1}) $\bar{X} \pm \text{SE}$
Baseline algae baseline oxygen	40.8 \pm 2.1	14.5 \pm 0.1	38.4 \pm 3.4	14.1 \pm 0.1
Baseline algae reduced oxygen	40.9 \pm 2.1	6.0 \pm 0.1	38.7 \pm 5.5	7.0 \pm 0.1
Reduced algae baseline oxygen	8.3 \pm 2.2	14.1 \pm 0.6	6.0 \pm 1.3	14.2 \pm 0.2
Reduced algae reduced oxygen	9.5 \pm 2.1	6.1 \pm 0.1	8.0 \pm 1.1	6.9 \pm 0.1

$n = 24$ for each treatment in the experiment on the effects on bitterling. $n = 11, 10, 9$ and 10 , respectively for the treatments in the experiment on the effects on mussels.

The 'reduced' dissolved oxygen concentration used in our experiments ranged from 6–7 mg l^{-1} . This was based on the mean concentration from 1990–1999 at Bramerton Woods End (River Yare), which is downstream from a sewage treatment works (UK Environment Agency data). The aim of our experiment was to investigate bitterling survival due to the reactions of their mussel hosts to changes in the environment. We therefore used low dissolved oxygen concentrations that were not expected to cause direct harm to freshwater fish embryos ($> 6 \text{ mg l}^{-1}$: Dourdoroff & Shumway, 1970; USEPA, 1986; Dean & Richardson, 1999).

The treatment oxygen concentrations were created using pre-determined ratios of nitrogen and oxygen bubbled into the tanks so that oxygen levels fell to the desired level within 2–3 h. A transparent plastic lid was placed over each aquarium to help maintain control. The oxygen concentration in each compartment was measured twice daily, using a dissolved oxygen meter, for the duration of the experiment. The differences in oxygen levels between the baseline and 'reduced' treatments for the bitterling experiments are shown in Table 1 (Kruskal–Wallis test: $H_{96} = 65.5, P < 0.001$).

Algae conditions

The two algal concentrations were created using pre-determined quantities of a live algal suspension derived from an outdoor pool that had been seeded initially with *C. vulgaris*. Algal concentrations were calculated using the concentration of chlorophyll *a* pigment in water extracted in acetone every 48 h following the method described by Mackereth, Heron & Talling (1978).

For the baseline algae treatments we used concentrations of 38–40 $\mu\text{g l}^{-1}$ of algae. This was based on the mean chlorophyll *a* concentration of $41.9 \pm 18 \mu\text{g l}^{-1}$ (mean \pm standard error (SE)) for April–July measured from 1996–1998 by the UK Environment Agency at a site 2.1 km upstream from our study site (Reach Lode, Hallards Fen Rd, Cambridgeshire, UK). Mean chlorophyll *a* concentrations of $25 \mu\text{g l}^{-1}$ represent eutrophic conditions in UK rivers (Anonymous, 1994), while $39.6 \mu\text{g l}^{-1}$ represents the average concentration for Bramerton Woods End (River Yare) downstream from

a sewage treatment works (UK Environment Agency data).

For the 'reduced' algae treatments we used concentrations of 6–9 $\mu\text{g l}^{-1}$ of algae. Mean chlorophyll *a* concentrations of less than $4 \mu\text{g l}^{-1}$ represent oligotrophic conditions (Mason, 1996b; Soto & Mena, 1999), and $8.2 \mu\text{g l}^{-1}$ represents the average chlorophyll *a* concentration for Trowse Mill (River Yare) upstream from a sewage treatment works (UK Environment Agency data). The differences between treatments in chlorophyll *a* concentrations for the bitterling experiments are shown in Table 1 (Kruskal–Wallis test: $H_{96} = 47.1, P < 0.001$).

Effect of treatments on mussel ventilation rates

Following the main experiment, four *A. anatina* mussels that had not been accessible to bitterling were distributed between the four compartments at baseline oxygen and baseline algae conditions. We performed 11 replicates of this experiment. The volume of water pumped per hour (ventilation rate; l h^{-1}), calculated from the velocity of water in the exhalant streams of mussels, was measured at two separate intervals over a 12 h period before the oxygen and algal treatment conditions were created and then afterwards every 48 h for 5 weeks. The valve position of each individual was also registered daily: (1) active, when siphons were visible and valves gaping and (2) inactive, when the valves were closed.

As with the previous experiment, we confirmed that the chlorophyll *a* concentrations in the baseline algae treatments were higher than those in the 'reduced' algae treatments (Kruskal–Wallis test: $H_{40} = 27.8, P < 0.001$; Table 1), and the oxygen concentrations in the baseline oxygen treatments were higher than those in the 'reduced' oxygen treatments (Kruskal–Wallis test: $H_{40} = 29.6, P < 0.001$; Table 1).

The velocity (flow speed; cm s^{-1}) of water in the exhalant streams of the mussels was determined using a small thermistor probe following the methods described by Mills & Reynolds (2002a). Mean rates at which mussels process the ambient water (ventilation rate, l h^{-1}) were calculated from flow speeds by simultaneously determining the cross-sectional area of the exhalant siphon

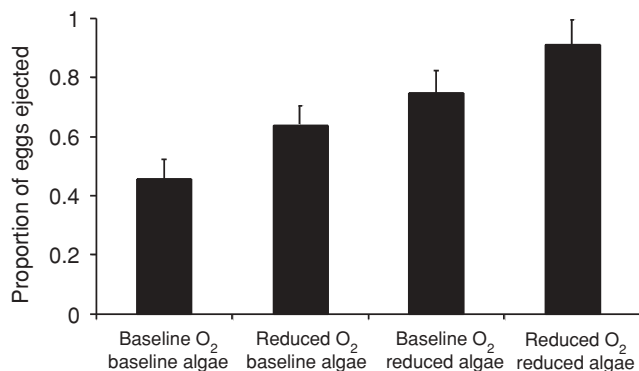


Fig. 1. Mean (+ standard error) proportion of embryos ejected by mussels in the four treatment conditions. $n = 24$ in all cases.

from a grid (0.5×0.5 mm) held next to the mussel's siphon (Mills & Reynolds, 2002a).

RESULTS

Effects of treatments on bitterling survival

The proportion of bitterling embryos that were ejected prematurely was significantly different between the different algae and oxygen treatments (Two-way ANOVA, algae: $F_{1,95} = 15.29$, $P < 0.001$; oxygen: $F_{1,95} = 5.71$, $P = 0.019$; Fig. 1). Both reduced algae and reduced oxygen increased the proportion of eggs ejected. The proportion of embryos ejected was not affected by the order of spawning preference (univariate model, $F_{3,93} = 0.11$, $P = 0.95$).

Bitterling embryos were ejected significantly more quickly from mussels in baseline algal than in 'reduced' algal treatments (mean \pm standard deviation (SD) of days to ejection: baseline O₂, baseline algae = 5.9 ± 0.5 ; reduced O₂, baseline algae = 5.0 ± 0.5 ; baseline O₂, reduced algae = 7.0 ± 0.5 ; reduced O₂, reduced algae = 6.9 ± 0.5 ; Two-way ANOVA, algae: $F_{1,91} = 7.27$, $P = 0.008$; oxygen: $F_{1,91} = 0.28$, $P = 0.60$).

Effect of treatments on mussel ventilation rates

As expected there was no difference in mussel ventilation rates among the four treatments before the conditions were applied (one-way ANOVA: $F_{3,37} = 0.07$, $P = 0.98$; Fig. 2). Also as expected, there was no difference in ventilation rates of mussels in the baseline O₂ and baseline algae treatment, both before and after the conditions were applied (Paired t test: $t_{11} = 1.45$, $P = 0.18$; Fig. 2). However, the ventilation rates of mussels in all of the other treatments were reduced after the conditions were applied (Paired t test: reduced O₂, baseline algae: $t_{10} = 2.9$, $P < 0.02$; baseline O₂, reduced algae: $t_9 = 5.1$, $P < 0.001$; reduced O₂, reduced algae: $t_{10} = 4.8$, $P < 0.001$; Fig. 2).

The ventilation rates of mussels were significantly different between the algal but not the oxygen treatments (Two-way ANOVA, algae: $F_{1,39} = 10.57$, $P = 0.002$; oxygen: $F_{1,39} = 1.71$, $P = 0.19$; Fig. 2). Reduced algae treatments significantly lowered mussel ventilation rates.

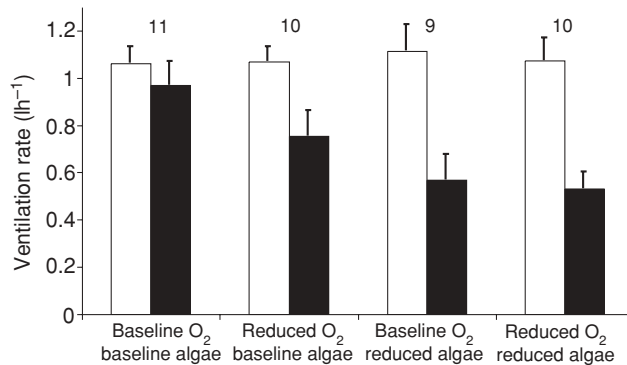


Fig. 2. Mean (+ standard error) ventilation rate of mussels in the four treatment conditions. Numbers above bars refer to sample size. □, before treatment; ■, after treatments were applied.

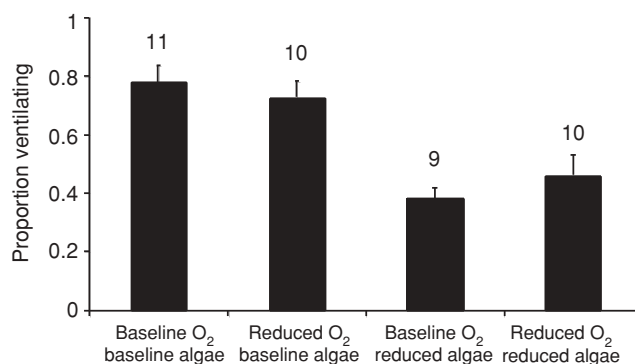


Fig. 3. Mean (+ standard error) proportion of observations that mussels' valves were found to be open and the mussels ventilating. Numbers above bars refer to sample size.

The proportion of observations during which mussels were active (valves open, siphons extended and mussels ventilating) was significantly different between the algal treatments, but not between the oxygen treatments (Two-way ANOVA, algae: $F_{1,39} = 33.87$, $P < 0.001$; oxygen: $F_{1,39} = 0.52$, $P = 0.82$; Fig. 3). Mussels were more active in the baseline algal treatments than in the reduced algal treatments.

DISCUSSION

This study has shown that as the concentrations of phytoplankton and dissolved oxygen decrease there is higher mortality of bitterling embryos due to premature ejection from mussels (Fig. 1), but only reduced algal conditions decreased mussel ventilation rates (Fig. 2) and valve activity (Fig. 3). Due to the symbiotic dependency of bitterling on mussels, the premature death of bitterling embryos may be due to either the direct effects of the environmental conditions, or to indirect effects through the reactions of their mussel hosts.

It is unlikely that the observed bitterling mortality was due solely to direct effects of the oxygen and algal conditions. For example, the higher mortality at reduced algal conditions would not have been due to bitterling starvation, since the embryos obtain their nutrients from

their yolk sac. Although we cannot rule out the possibility that mortality under reduced oxygen was due to direct effects on the embryos, the oxygen concentrations were above those known to cause impairment to embryos of other species of freshwater fish (Dean & Richardson, 1999). Instead, it is more probable that bitterling died from indirect environmental effects due to adverse conditions inside the hosts, created by their hosts in response to environmental changes. Mussels reduced their ventilation rates in reduced algal conditions (Fig. 2). This finding agrees with other studies of freshwater mussels (Badman, 1975; Famme & Kofoed, 1980; Sobral & Widdows, 1997). If the gill oxygen concentration fell well below 5 mg l^{-1} , this may have killed bitterling embryos. Gill oxygen concentrations may have been lowered further as a result of mussel respiration as well as by the consumption of oxygen by the bitterling embryos themselves (Smith *et al.*, 2001). Therefore, environmental conditions may have indirectly caused premature bitterling death by reducing mussel ventilation rates and thus the rate at which oxygenated water was available to the bitterling embryos.

The increased closure of mussel valves in reduced algae conditions (Fig. 3) may also be responsible for the higher bitterling mortality observed in the two reduced algae treatments and for the longer time taken for the embryos to be ejected, since the time to ejection may have been delayed by the closed valves. Valve closure stops water transport and oxygen delivery and accumulates anaerobic and excretory products, which are all detrimental to embryonic survival (Famme & Kofoed, 1980; Herreid, 1980; Bayne & Newell, 1983; Massabuau, Burtin & Wheathley, 1991). Valve closure is considered to be a general response by bivalves to environmental stressors (Sloof, de Zwart & Marquenie, 1983; Kramer, Jenner & de Zwart, 1989; Reynolds & Guillaume, 1998) and studies have shown that freshwater mussels escape food deprivation and low oxygen with shell closure (Salánki, 1964; Badman, 1974; Heinonen *et al.*, 1997; Sobral & Widdows, 1997). Mussels also remain closed in metabolic dormancy under prolonged adverse conditions (Storey & Storey, 1990; Hand, 1991) due to their ability to tolerate environmental anoxia (Holopainen & Penttinen, 1993). Unionid mussels, in particular, are highly tolerant compared with other freshwater bivalves (Matthews & McMahon, 1999). These behavioural adaptations of mussels may further compromise the survival of developing bitterling. Whereas most fish species with parental care increase fanning under adverse conditions such as low oxygen (e.g. Jones & Reynolds, 1999), mussels close their valves and tolerate anoxia until conditions improve. We suggest that this behaviour may be one of the reasons why bitterling have disappeared from some polluted European areas in which mussels are still present (Lelek, 1980). The reduction in algal diversity associated with eutrophication (e.g. Mason, 1996b) may also be important to mussel and, therefore, bitterling survival, since any shift towards algal species that cannot be assimilated by mussels will have even more severe consequences for bitterling.

Bitterling embryos may also have been affected indirectly by the algal and oxygen conditions through the reactions of their mussel hosts in a different way. Bitterling embryos are 2–3 mm in diameter and an average mussel would have contained 6–8 of them based on two spawnings and 3–4 eggs deposited per spawning (Mills & Reynolds, 2002b). Therefore, the presence of bitterling embryos in a mussel's water tubes would almost certainly obstruct the water flow, in a similar way to a mussel's own larvae (glochidia) in gravid gills (Tankersley & Dimock, 1993b). Mussels may have benefited from ejecting bitterling from their gills, thus increasing the surface area of the gills for oxygen uptake and filter feeding. This behaviour may become more frequent when oxygen and algae conditions decline and it is well documented that female unionid mussels abort their own larvae (glochidia) prematurely under conditions of low oxygen (e.g. see Tankersley & Dimock, 1993a). Alternatively, bitterling embryos may have been ejected as a side-effect of other mussel behavioural reactions to declining algae and oxygen conditions. We cannot verify or refute this theory because although the embryos found outside the mussels were dead, some time may have elapsed between their ejection and their detection, so we do not know whether they were alive or dead on leaving the mussel.

Our results show that two key effects of eutrophication – low oxygen and high phytoplankton levels – have opposing effects on bitterling embryo survival. Bitterling mortality is lowest and mussel ventilation rates are highest in high algal conditions, but mortality is also lowest in high oxygen conditions. These results provide an example whereby a species in symbiosis is particularly vulnerable to environmental changes through impacts on their hosts and their hosts' behavioural responses. Such interactions between species complicate conservation management, since a sub-lethal effect on one species can be translated into a lethal impact on another. For example, toxic chemicals and trace metals are more toxic to parasites, such as intestinal helminths and tapeworms, than to their hosts, due to the hosts' physiological concentrating effects (Riggs, Lemly & Esch, 1987; Lafferty & Kuris, 1999). Thus, we suggest that habitat restoration programmes in the bitterling's native European range do more than conserve bitterling, but also create habitats that are optimal for mussels. This recommendation echoes the point made recently by Redford & Feinsinger (2001), about the importance of preserving species interactions in order to avoid long-term deterioration of ecosystems.

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