# Conservation ecology of the endangered freshwater pearl mussel, <italic>Margaritifera margaritifera</italic>.

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# Conservation Ecology of the Endangered Freshwater Pearl Mussel, *Margaritifera margaritifera*

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## ABSTRACT

The general aim of this thesis was to examine the merits of *ex-situ* vs. *in-situ* strategies for the conservation of the endangered freshwater pearl mussel, *Margaritifera margaritifera*, and to investigate the relationship of the larval parasitic stages of the mussel (glochidia) with the salmonid hosts. To this end, I critically reviewed the literature on conservation of freshwater mussels, developed methods for quantifying the behaviour and activity patterns of adult mussels in captivity, experimentally studied host specificity, and quantified the physiological and behavioural effects of glochidia upon salmonid hosts. The results indicate that the conservation of the freshwater pearl mussel is probably best addressed at the watershed scale, and will benefit from a combination of *ex-situ* and *in-situ* techniques, as well as from a more critical assessment of findings, many of which are only reported in the grey literature. Empirical, peer-reviewed data are badly needed to inform current conservation efforts. Novel Hall-effect magnetic sensors were used to quantify and characterise discrete mussel behaviours without adversely affecting the welfare or survival of adult mussels, and these hold considerable potential for determining optimal rearing conditions for *ex-situ* conservation. Arctic charr was shown to be a potentially suitable host for *M. margaritifera,* and occupied an intermediate position in host suitability between brown trout and Atlantic salmon. Physiological impacts of glochidia upon brown trout included swelling of secondary lamellae and spleen enlargement, but the latter tended to be slight and was restricted to 1 month post-exposure. Glochidia encystment had no significant effect on blood haematocrit, respiratory performance, or cryptic colouration of brown trout hosts. The behavioural effects were more subtle and glochidiosis made brown trout more risk-averse and less willing to explore a novel habitat, without affecting the host's ability to chemically recognise and avoid cues from a predator. Overall, the results of this thesis indicate that the impacts of glochidia upon salmonid hosts are probably slight and temporally variable, and may perhaps lead to increased host survival, which would support the symbiosis-protocooperation theory of glochidia-salmonid interaction.

DECLARATION

This work has not previously been accepted in substance for any degree and is not concurrently submitted in candidature for any degree

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STATEMENT 1

This thesis is the result of my own investigations, except where otherwise stated. Where correction services have been used, the extent and nature of the correction is clearly marked in a footnote(s).

Other sources are acknowledged by footnotes giving explicit references. A bibliography is appended.

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STATEMENT 2

I hereby give consent for my thesis, if accepted, to be available for photocopying and for interlibrary loan, and for the title and summary to be made available to outside organisations.

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# CONTENTS

**CYDNABYDDIAETHAU**

Ni fysai'r thesis hyn wedi bod yn bosibl heb chymorth, arweiniad a goruchwyliaeth Dr. Carlos Garcia de Leaniz. Yr wyf yn hynod o ddiolchgar iddo am ei gefnogaeth a chyngor amhrisiadwy ac ysbrydoledig. Rwy'n credu fy mod wedi datblygu fel gwyddonydd a pherson gwell o ganlyniad i'r gwaith hwn, ac mae'r clod am hyn yn perthyn i Carlos.

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# Disclaimer

**Chapter I:** Literature analysis and writing of the manuscript were conducted by Gethin Rhys Thomas (GRT). Dr. Carlos Garcia de Leaniz (CGL) supervised and contributed to the manuscript. Dr. Sonia Consuegra (SC) and Dr. John Taylor (JT) provided comments on the manuscript, as did three anonymous referees.

**Chapter II:** Literature was analysed and the manuscript written by GRT. Supervision and contributions to the manuscript were provided by CGL.

**Chapter III:** Data collection, analysis and manuscript creation were carried out equally by Dr. Anthony Robson (AAR) and GRT. CGL and Prof. Rory Wilson (RW) contributed to the manuscript and provided supervision. Dr. Nikolai Liebsch (NL) provided technical assistance. Three anonymous referees provided comments to the final manuscript.

**Chapter IV:** Data collection and analysis were conducted by GRT. Prof. Andrew Rowley (AR) assisted with histological techniques and analysis. The manuscript was written by both GRT and CGL.

**Chapter V:** GRT conducted data collection and analysis. CGL supervised and provided comments and contributions to the manuscript.

**Chapter VI:** GRT conducted data collection and analysis. Dr. Laura Roberts (LR) assisted with designing behavioural experiments. CGL supervised and provided comments and contributions to the manuscript.

# INTRODUCTION

## Freshwater mussels – an imperilled taxon

Freshwater pearl mussels (Bivalvia: Unionoida) rank among the most endangered aquatic organisms in the world (Strayer et al 2004). There are several families of freshwater mussels in the Unionoida, and all have an obligate parasitic stage in their lifecycle, which requires encystment on a suitable host fish in order to complete their development. The most widespread species of the family Margaritiferidae is *Margaritifera margaritifera*, with a Holarctic distribution ranging from the Iberian Peninsula (40°N) to Arctic Russia (70°N). With a maximum life span in excess of 150 years, freshwater pearl mussels rank amongst the slowest growing and longest-lived known invertebrates (Ziuganov et al 2000; Anthony et al 2001). This species has also suffered the steepest decline of all extant freshwater mussel species (Young et al 2001; Hastie et al 2003). *M. margaritifera* is strictly protected in most countries, including the UK, which holds (in Scotland) possibly more than half of the world's remaining reproducing populations, although large populations also occur in Scandinavia (Cosgrove et al 2000; Young et al 2001). There are several causes for the decline in *M. margaritifera*, including water pollution, increased siltation, overfishing and the collapse of host fish populations, illegal pearl fishing and construction of dams (Young & Williams 1983; Watters 1996; Vaughn & Taylor 1999; Cosgrove et al 2000; Morales et al 2004). All of the known causes for the decline of this species are as a result of human activities.

The accelerated decline of many freshwater mussels has resulted in a range of initiatives designed to conserve these species in Europe (Buddensiek 1995: Beasley & Roberts 1999; Hastie & Young 2003; Preston et al 2007) and elsewhere (Strayer et al 2004; Barnhart 2006). Sometimes, entire *M. margaritifera* populations have been collected from the wild and brought into captivity in the hope of establishing living gene banks and aid in the recovery of self-sustaining populations (Thomas et al 2010). Despite this recent focus, there is a notorious paucity of data on critical life stages and the relative merits of different conservation strategies. The risk with captive breeding programmes is that resources may be diverted away from habitat restoration and improvement, without guarantee of success. Many of the underlying stressors affecting freshwater mussels relate to whole catchment processes, which

tend to be very difficult to address (Strayer 2008). Habitat improvement (e.g. improving water quality, reducing silt loads, restoring river connectivity and maintaining minimum flows) should in theory benefit the conservation of freshwater mussels (Beasley & Roberts 1999; Cosgrove & Hastie 2001; Poole & Downing 2004) but there are no long term data on the success of such measures. Given that resources allocated to mussel conservation are always likely to be limited, it is essential to weigh and prioritize the different options available to freshwater managers and wildlife officials (Araujo & Ramos 2001), an aspect that I examine in **Chapters I and II**.

## Conservation challenges

The conservation of *M. margaritifera* is particularly problematic as it is exacerbated by the continuation of many practices and activities that actively contribute to its decline. Many mussel populations display a skewed age ratio (Araujo & Ramos 2001; Skinner et al. 2003), with an overrepresentation of older individuals which may not be reproducing. It has been argued that until the situation in rivers improves, the conservation of this species will have to rely on *ex-situ* conservation (captive breeding). Whilst initially appealing due to its dramatic and highly visible methods, *ex-situ* conservation alone is rarely an effective way to safeguard a species from extinction (Snyder et al 1996). For example, despite repeated large scale re-introductions of Atlantic salmon across its historical range, this species has not become re-established (Marttunen & Vehanen 2004). Captive breeding programmes often fail to achieve their objectives because stocked animals compete poorly with wild counterparts as a result of different selective forces acting within *ex-situ* and *in-situ* environments (Naish et al 2008). The *ex-situ* conservation of *M. margaritifera* will depend, as with all *ex-situ* conservation strategies, on the ability of captive-bred individuals to survive and reproduce in the natural environment, not on the success of the rearing programme itself. However, there remain large gaps in our knowledge of *M. margaritifera* biology, and few studies have specifically addressed the conservation of freshwater mussels (Thomas et al 2010). For example, despite several European populations of *M. margaritifera* having been removed from rivers and maintained in captivity, there remains uncertainty over the dietary or habitat

requirements of adult mussels (Robson et al 2009; Thomas et al 2010), and it remains unknown whether captive populations will adapt to the natural environment once released, or how hatchery-reared juvenile mussels fare compared to wild counterparts. For this reason, we employed newly developed Hall-sensor technologies (Robson et al 2009) to examine in detail the behaviour of adult freshwater mussels, as discussed in **Chapter III.** Our aim was to develop a reliable, non-intrusive way of quantifying the activity patterns and welfare of adult mussels held in captivity, in an effort to design better *ex-situ* conservation methods.

## A very specific species?

Like all unionid mussels, the larvae of *M. margaritifera* (termed glochidia) are obligate gill parasites of fish. Glochidia encyst onto the gill lamellae of a suitable host fish and develop for several months before they drop off into a suitable substrate (Hastie & Young 2003). Although glochidia can readily attach to the tissue and gill filaments of various fish species (Strayer et al 2004), metamorphosis and full larval development is normally only possible on a few host species (Dodd et al 2006). Margaritiferids appear to be extremely host-specific, being closely linked to non-migratory brown trout (*Salmo trutta*) and migratory fishes (salmonids in the case of *M. margaritifera* and Acipenserids in the case of *M. auricularia* (Altaba 1990; Araujo et al 2001; Lopez et al 2007). This high degree of specificity is demonstrated by the inability of *M. margaritifera* to successfully encyst on Pacific salmonids (Meyers & Millemann 1977; Young & Williams 1984; Bauer 2000; Skinner et al. 2003).

The host-parasite relation between salmonid hosts and *M. margaritifera* can be considered as a good system to examine local adaptations at the more controversial end of the host-parasite continuum (parasites with longer generation time than the host) because (a) the salmonid hosts' shorter generation time and migratory behaviour will tend to favour the development of localised host adaptation (LHA), while (b) the parasite's (mussel) narrow host range will tend to favour the development of localised parasite adaptation (LPA). It is also a good system to understand adaptive responses to environmental uncertainty and climate change (Hastie et al 2003) since the host can move but the parasite cannot.

Local parasite adaptation appears to be common on plant-invertebrate systems with limited host dispersal and/or relatively short parasite generation times, but whether LPA is also the norm in other systems with long parasite generation times or highly dispersive hosts is subject to debate (Gandon & Michalakis 2002; Lajeunesse & Forbes 2002). Recent declines in both *M. margaritifera* and Atlantic salmon (two of the most endangered aquatic organisms in Europe, Hastie & Young 2003; Young et al 2001) stress the need for knowledge on the precise nature of the interaction between *M. margaritifera* and its hosts.

The potential for localised adaptations by both mussel and host fish may be of relevance to conservation strategies that rely on *ex-situ* methods such as captive breeding or stocking of infected hosts (Thomas et al 2010). It has been suggested that attempts to conserve declining populations of *M. margaritifera* should include a consideration of the interactions between these mussels and their salmonid hosts (Geist et al 2006; Geist 2010) as uncertainty remains regarding host specificity in *Margaritifera margaritifera* even at the species level. This provided the rationale behind the host specificity studies detailed in **Chapter IV**, whereby the responses of three salmonid species to glochidia exposure were quantified in a 'common garden' exposure experiment.

## Glochidia–host interactions

The responses of salmonids to *M. margaritifera* infection are poorly known (Treasurer & Turnbull 2000; Treasurer et al 2006). Mortalities of juvenile salmonids have been reported following artificial glochidia infection, and hatchery losses have sometimes been attributed to glochidiosis (Meyers & Millemann 1977; Treasurer et al 2006). Yet, there is limited information on the impacts of glochidia on their hosts, despite the fact that the parasitic stage is an essential component for the development of effective conservation programmes (mostly based on the artificial infection of salmonid hosts in captivity). Freshwater mussel glochidia must remain attached to their hosts for varying periods of time in order to complete their development. Over the course of the encystment, fish mount an immune response specifically targeting the glochidia (Meyers et al 1980; Bauer & Vogel 1987; O'Connell & Neves 1999),

which results in the shedding of large numbers of the parasites (Hastie & Young 2003).

As the host mounts an immune response against glochidia, it can be assumed that glochidiosis presents the host with a burden, whereby it is advantageous to remove as many glochidia as possible. The development of "acquired immunity" against glochidia, first noted by Reuling (1919), and confirmed in both the *M. margaritifera*-salmonid and other similar systems (Fustish & Millemann 1978; Bauer 1987; Bauer & Vogel 1987; Rogers-Lowery et al 2007) also supports the contention that it must be advantageous for fish to rid themselves of glochidia. However, as obligate parasites, the fate of encysted glochidia is inexorably linked to that of the host; if during the course of encystment the fish dies, then so do glochidia. Whilst some trophically-transmitted parasites have been shown to alter the behaviour and physiology of the host to make it more likely to be preyed upon (e.g. Barber et al 2000; Mikheev et al 2010), very little is known about the effects of non-trophically transmitted glochidia. Salmonids are obligate, definitive hosts of the glochidia of *M. margaritifera*; therefore trophic transmission (through predation on the host) is not necessary. On the contrary, predation of encysted hosts is to be avoided if the mussel is to survive to the next stage. It was therefore hypothesized in this thesis that for parasitic glochidia to develop successfully in the host (a process lasting several months) it might be advantageous to make the host more risk-averse, thereby reducing the likelihood of predation. Indeed, the relationship between *M. margaritifera* and its hosts has been proposed to be an example of symbiosis-protocooperation (Ziuganov & Nezlin 1988; Geist 2010), although no studies have experimentally tested this hypothesis. The experiments detailed in **Chapters V** and **VI** examined the physiological and behavioural responses of brown trout to glochidia encystment, and attempted to quantify temporal changes in host responses.

# Aims and Objectives

The overall aim of this thesis was to further knowledge on critical aspects of freshwater mussel biology and conservation, namely *ex-situ* and *in-situ* conservation methods, and to analyse the nature of the interactions between glochidia and their fish hosts. It was hoped that such understanding would enable environmental officers to improve the efficiency of conservation programmes for *M. margaritifera*. To achieve this end, I first conducted a critical appraisal of the merits of *ex-situ* vs. *in-situ* conservation approaches for freshwater mussel conservation (Chapters I - II), studied the activity and behaviour of adult mussels in captivity (Chapter III), assessed the extent of host specificity (Chapter IV), and quantified the physiological (Chapter V) and behavioural (Chapter VI) responses of salmonid hosts to glochidia encystment (Plate 1).

## Chapter outline

This thesis consists of six chapters, two of which have already been published (Chapter I: *Endangered Species Research* **12**, 1-9; Chapter III: *Aquatic Biology* **6**, 191-200); Chapter IV is under review (*Freshwater Biology*), and chapters III, V and VI are in preparation for peer-review submission. Appendix I at the end of the thesis includes details of the methods I employed for histological examination of fish host tissues.

***Chapter I: Captive breeding of the endangered freshwater pearl mussel Margaritifera margaritifera***

This critical review of the published literature was undertaken to establish the current methods in the captive rearing of freshwater mussels. Several *ex-situ* conservation methods had been developed (e.g. Buddensiek 1995; Hastie & Young 2003; Preston et al 2007) in an attempt to breed mussels in captivity, but there was no review of the effectiveness and applicability of various approaches. The aim of this review was, thus, to collate and critically assess the merits of various *ex-situ* conservation methods for *M. margaritifera* and other freshwater mussels, to identify gaps in knowledge, and to provide suggestions for future research and improvement of captive breeding efforts.

The question asked in this chapter was therefore:

*What are the current methods in ex-situ conservation of freshwater mussels, and what are their relative merits and drawbacks?*

***Chapter II: In-situ conservation of the freshwater pearl mussel Margaritifera margaritifera***

The aim of this review was to assess the published data on various *in-situ* conservation strategies, and to provide a critical assessment of the effectiveness of such methods. Much of the restoration of freshwater mussel carried out by government agencies does not enter the primary literature and is seldom monitored. Consequently, our understanding of *in-situ* conservation methods is incomplete and fragmentary, and a review of techniques was needed. This chapter illustrates the range of options available for *in-situ* conservation of freshwater mussels, and considers the relative merits and limitations of various restoration strategies. The questions I asked in this chapter were:

*What methods exist for in-situ conservation of freshwater mussels, and how could these methods be best applied to the conservation of* Margaritifera margaritifera*?*

***Chapter III: Monitoring the behaviour of the endangered freshwater pearl mussel Margaritifera margaritifera (Bivalvia: Unionidae): conservation applications***

With the growing development of captive breeding programmes, more and more mussels are being removed from their habitats and kept in captivity (Thomas et al 2010). However, little if any attention has been given to the welfare of adult broodstock whilst in captivity, despite current understanding that bivalve behaviour is both complex and subtle. This hatchery- and laboratory-based study aimed to develop methods and technologies suitable for quantifying the activity patterns and welfare of various bivalve species. The questions I asked were:

*Can novel technologies be used to record bivalve behaviour without compromising the welfare and survival of endangered species, and what are the possible applications of such methods?*

***Chapter IV: Ghosts of hosts past – host specificity in the endangered freshwater pearl mussel Margaritifera margaritifera***

Understanding host responses to glochidiosis and the susceptibility of different fish hosts to *M. margaritifera* is key to understanding the ecology of the freshwater pearl mussel. Yet, few studies have quantified host responses to glochidia, or how such responses may vary amongst fish hosts. In this study three salmonid species were exposed to the glochidia of a single population of *M. margaritifera* using a common garden approach, and their responses to glochidiosis at a single point in time were quantified and compared. In this chapter, I asked the following questions:

*What is the extent of host specificity in* M. margaritifera*? And how do different salmonid species respond to glochidiosis?*

***Chapter V: Temporal variation in the physiological responses of brown trout to the glochidia of Margaritifera margaritifera***

This laboratory-based study compared the physiological responses of a single salmonid species, brown trout, to glochidiosis at various times post-exposure. Few studies have investigated the physiological impacts of glochidia on their host, and some results are contradictory. This study addressed two questions:

*What are the physiological effects of glochidia on brown trout? How do these effects change over the course of infection?*

***Chapter VI: Backseat driving: behavioural effects of Margaritifera margaritifera***

The effects of trophically-transmitted parasites on fish behaviour have been studied, and the evolutionary significance of any behavioural changes on the host that can facilitate parasite transmission are generally well understood (e.g. Barber et al 2000). However, there are no studies on the behavioural response of fish hosts to the non-trophically transmitted glochidia, despite the fact that host behaviour is a critical determinant of survival of both host and parasite. In this laboratory-based study I asked the following questions:

*Do glochidia have an effect on host behaviour, and if so, how could this effect influence host survival and glochidia encystment success?*

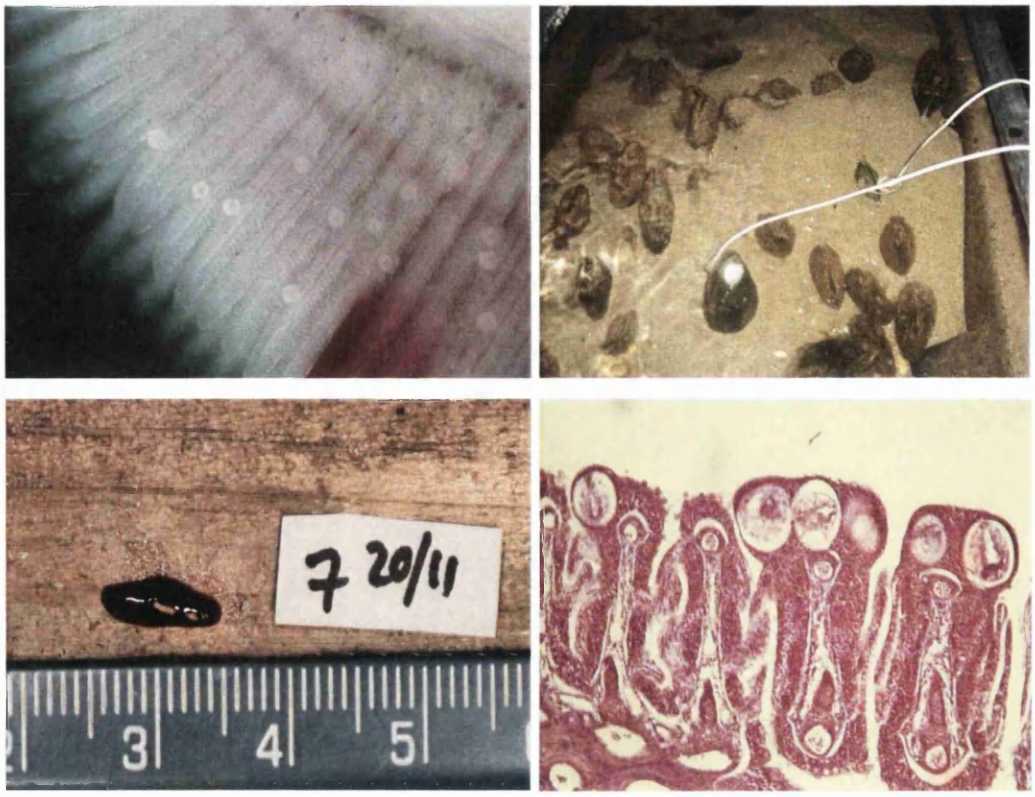


**Plate 1.** Glochidia encysted on brown trout gill (Chapter V, top left); *M. margaritifera* with attached Hall sensor (Chapter III, top right); dissected spleen from brown trout (Chapter V, bottom left); histological section through an Arctic charr gill showing encysted glochidia (Chapter IV, bottom right).

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# Chapter I.

## Captive Breeding of the Endangered Freshwater Pearl Mussel, *Margaritifera margaritifera*

Thomas, G.R., Taylor, J., Garcia de Leaniz, C. (2010) Captive breeding of the endangered freshwater pearl mussel *Margaritifera margaritifera*. *Endangered Species Research* **12** (1): 1-9

# Chapter I.

# Captive breeding of the endangered freshwater pearl mussel *Margaritifera margaritifera*


## ABSTRACT

Freshwater pearl mussels (Unionidae: Bivalvia) rank among the most endangered aquatic invertebrates, and this has recently prompted a number of initiatives designed to propagate the species through captive breeding. Yet there are few guidelines to aid in freshwater mussel culture for conservation, and few or no results on the fate of released juveniles. Here we review various *ex-situ* strategies for freshwater mussel conservation with emphasis on the freshwater pearl mussel (*Margaritifera margaritifera* L.), one of the most critically endangered unionids. Captive breeding could help safeguard critically endangered populations, but current rearing methods need to be optimised. Areas in particular need of research include the collection and storage of viable glochidia, the development of efficient rearing systems, and the formulation of algal diets. Likewise, the degree of host specificity warrants further investigation, as this will largely dictate the success of reintroduction programs. Finally we note that more information is needed on the degree of genetic structuring and post-release survival before translocation programs can be recommended. As with other conservation projects, captive breeding of the freshwater pearl mussel cannot compensate for loss of critical habitats and is likely to be most efficient in combination with *in-situ* conservation, not in isolation.



**Keywords**: Freshwater pearl mussel, *Margaritifera margaritifera*, captive breeding, host specificity, juvenile culture

## INTRODUCTION

Freshwater pearl mussels (Unionacea) are among the most endangered aquatic organisms in the world (Strayer et al. 2004). With a maximum life span in excess of 100 years, some pearl mussels also rank among the slowest growing and longest living known invertebrates (Ziuganov et al. 2000, Anthony et al. 2001), which makes their conservation particularly problematic (Cosgrove & Hastie 2001, Hastie et al. 2003). The accelerated decline of many freshwater mussels has recently prompted a flurry of initiatives designed to propagate and restore the species in Europe (Buddensiek 1995, Beasley & Roberts 1999, Hastie & Young 2003a, Preston et al. 2007) and elsewhere (Strayer et al 2004, Barnhart 2006). In the UK, unprecedented steps have recently been taken to safeguard entire *M. margaritifera* populations by collecting adults from the wild and bringing them into captivity in the hope of establishing living gene banks and aid in the recovery of self-sustaining populations (Taylor 2007). Yet, there is a paucity of data on critical life stages, the relative merits of different conservation strategies, or the fate of cultured juveniles.

Given that resources allocated to mussel conservation are always likely to be limited, it is essential to weigh and prioritize the different options available to freshwater managers and wildlife officials (Araujo & Ramos 2001). Whilst the *in-situ* requirements of different freshwater mussel species have already been discussed by others (Neves & Widlak 1987, Layzer & Madison 1995, Valovirta 1998, Hastie et al 2000, Brainwood et al 2008), few guidelines exist for *ex situ* conservation. Here we critically review various strategies for the *ex-situ* conservation of the freshwater pearl mussel, examine the main gaps in knowledge, and indicate those areas in most need of research. Although we have largely focused our attention to the freshwater pearl mussel we have also drawn information from other freshwater mussels, where appropriate. Our objectives were twofold: (1) to illustrate the range of options available for the artificial propagation of freshwater mussels, and (2) to weigh the main advantages and limitations of different captive breeding strategies for conservation.

## STRATEGIES FOR *EX SITU* CONSERVATION

The conservation of *M. margaritifera* faces several challenges, not least being the low rates of recruitment in natural populations. This is offset by a long reproductive lifespan and high fecundity, but it still takes 10-20 years for adult freshwater pearl

mussels to become sexually mature (Bauer 1987a, Skinner et al. 2003). *Ex situ* conservation of freshwater pearl mussels involves some or all of the following steps (Figure 1.2): (1) fertilization of females in captivity, (2) infection and encystment of glochidia in suitable fish hosts, (3) stocking of infected fish into existing or historical mussel rivers, (4) harvesting and rearing of excysted larvae, and (5) release of captive-reared juvenile mussels. Historically, *ex-situ* conservation projects have on the whole been uncoordinated and poorly planned, with results difficult to quantify due to the slow turnover of this species (Hastie & Young 2003a).

**Fertilization of females in captivity**

Mussel fertilization rates are known to be influenced by the spatial distribution of broodstock (Downing et al. 1993), and the aim of aggregating adult mussels in captivity is to achieve higher fertilization rates and greater production of glochidia. In common with other freshwater bivalves, sexes in the freshwater pearl mussel are separate (dioecious) and reproduction takes place after 10-20 years, typically in February or March (Young & Williams 1984a,b, Skinner et al. 2003). Males release sperm into the water, which is carried downstream and inhaled by females to fertilize their eggs, kept in modified marsupia in the gills (Smith 1979, Skinner et al. 2003). Fertilization often occurs synchronously within a population, and appears also to be linked to water temperature (Ross 1992, Buddensiek 1995, Hastie & Young 2003b), as in other species of freshwater mussel (Watters & O'Dee 1999). At low densities, females can turn hermaphroditic, but whether this results in self-fertilisation is not clear (Bauer 1987b, Hanstén et al. 1997).

It is as yet unclear how many adults are required to achieve a reproductively viable population in captivity. In Wales, the Freshwater Pearl Mussel Recovery Group advocated in 2005 the collection from the wild of all adult mussels in the most critically endangered populations (those consisting of fewer than 100 mussels), and the rearing in captivity of at least 50 adult mussels from each of the other populations (Taylor 2007). Adult mussels have been kept in flow-through systems fed with river water or in re-circulating systems. In flow-through systems, mussel broodstock can be maintained in salmonid hatchery troughs supplied with filtered river water (30 μm) to reduce sediment loads, and covered with sand and gravel (Hastie & Young 2003a, Preston et al. 2007). Very little is known about the diet requirements of adult *M. margaritifera*, although information from other freshwater

bivalves suggests that they probably feed on freshwater algae within the 15- 40 μm range (Winkel & Davids 1982). In the wild, Mandal et al (2007) found varying proportions of blue-green algae, green algae and diatoms in the gut of the freshwater mussel *Lamellidens marginalis*. Mussels kept in recirculating systems need to be fed with a suitable algal diet, but it is unclear whether or not supplemental feeding is needed in flow-through systems, or what effects - if any - different diet may have on reproduction and gamete quality. Recent research on stable isotope composition of mussel shells (Geist et al 2005) may assist in the formulation of suitable diets for captive mussels.

**Infection of fish hosts and host specificity**

Although glochidia of most Unionid mussels can readily attach to the tissue and gill filaments of various fish species (Strayer et al. 2004), metamorphosis and full larval development is normally only possible on a few host species (Dodd et al. 2006). In the case of *M. margaritifera*, each female can release between 1 million and 4 million glochidia, which drift downstream and die within 24-48 hr. if they cannot attach to a suitable fish host (Hastie & Young 2003b), although in some cases can remain infective for up to six days (Ziuganov et al. 1994, Skinner et al. 2003). Margaritiferids appear to be highly host-specific, being closely linked to non-migratory brown trout (*Salmo trutta*) and migratory fishes (salmonids in the case of *M. margaritifera* and acipenserids in the case of *M. auricularia* (Altaba 1990, Ziuganov et al. 1994, Bauer 2000). The Atlantic salmon (*Salmo salar*) is thought to be the primary fish host for *M. margaritifera* across its range (Ziuganov 2005), although brook trout (*Salvelinus fontinalis*) in eastern N. America and brown trout (*Salmo trutta*) in Europe can also act as suitable hosts (Young & Williams 1984b, Bauer 1987a, 2000, Cunjak & McGladdery 1991, Hastie & Young 2001, 2003b, Morales et al. 2004). There is also a suggestion that Artic charr (*Salvelinus alpinus*) may act as a viable fish host in northern Europe (Bauer 1987a), but this has not yet been confirmed (Hastie & Young 2001). Walker (2007) notes that, although rare, *S. alpinus* coexists in rivers with *M. margaritifera* in Scotland, providing the opportunity for glochidia to encyst on this species.

What seems clear is that *M. margaritifera* cannot metamorphose in the gills of Pacific salmonids (Young & Williams 1984a, Bauer 2000, Skinner et al. 2003, Ziuganov 2005). Earlier accounts on the susceptibility of Pacific salmonids to *M.*

*margaritifera* in western North American (Meyers & Millemann 1977) are now believed to refer to the closely related species *M. falcata* (Stone et al 2004), and may explain the contradictory results. Table 1.1 summarizes the known hosts of *M. margaritifera* across its range. The extent to which freshwater pearl mussels show intraspecific variation in host specificity is not known and warrants further study as this may dictate the success of reintroduction programs.

**Encystment of glochidia**

Perhaps the simplest way to achieve host encystment of glochidia is by making gravid mussels cohabit with juvenile salmonids in hatchery troughs (Treasurer et al. 2006). Typically 0+ salmonid fry are used (either Atlantic salmon or brown trout) to maximize encystment, as older salmonid parr may show acquired immunity from previous exposures (Treasurer et al. 2006). Rearing salmonids and mussels together appears to result in high encystment rates (Treasurer et al 2006), and it is possible that the release of glochidia in *M. margaritifera* is facilitated by the close proximity of suitable fish hosts, as shown in other freshwater mussels (Haag & Warren 2000). Research on the role of fish hosts in triggering *M. margaritifera* spatting would seem warranted in order to optimize captive breeding programs.

As an alternative to the cohabitation method, the outflow of tanks housing gravid mussels can be diverted into fish tanks housing hatchery-reared juvenile salmonids (Hastie & Young 2003a, Preston et al. 2007). Hastie & Young (2001, 2003a) showed that large numbers of Atlantic salmon and brown trout could be infected in this way, with glochidia loads ranging between 10 and 800 glochidia per fish. More recently, Preston et al. (2007) used the same approach to infect large numbers of juvenile brown trout with low (~1%) host mortalities.

In captivity, released glochidia which do not find their way into fish hosts can often be observed as a white, dense cloud in or around the adult female. This can be collected, diluted if necessary and either poured directly into hatchery tanks, or be given as a bath to batches of fish in small volumes of water to achieve infection. Spatting can also be induced in captivity, when it does not occur naturally. To induce glochidia release, gravid females are first placed in chilled de-chlorinated tap water. The release of glochidia is usually observed within 1 hour as water rises to room temperature (Meyers & Millemann 1977). Induction of spatting is believed to be caused by thermal shock and respiratory stress, resulting in the forced release of

glochidia from the modified gill marsupia to reduce oxygen demand; more oxygen becomes available to the female after expelling the brooding glochidia (Hastie & Young 2003b). Glochidia are then examined for viability, with cilia movement and 'winking' of valves as viability criteria; various salt concentrations can also be used to elicit an open/close response to determine glochidia viability (Meyers & Millemann 1977). Only glochidia spawned on the same day are normally used. The use of induced glochidia allows better control over exposure concentrations, but it is not known to what extent this method compromises glochidia viability compared to those obtained from naturally spawned mussels. Indeed, spat induced by thermal shock have sometimes been found to consist of immature, non-viable glochidia.

**Stocking of infected fish hosts**

The release of artificially infected hosts into rivers has a long history (Buddensiek 1995, Valovirta 1998, Hruska 2001, Preston et al. 2007), though results have been difficult to quantify. In Germany, and more recently also in the British Isles, there have been large releases of infected salmonid hosts, but evidence for recruitment of second generation juvenile mussels is lacking (Hastie & Young 2003a). In theory, the release of artificially infected hosts makes conservation sense, as the maturing glochidia would fall from the host and populate the rivers in a 'natural' way, and would also reduce the costs and time associated with an extended period of juvenile mussel rearing in captivity. Moreover, artificial infection typically results in glochidia loads many times higher than those commonly found in the wild (Karna & Millemann 1978, Hruska 2001), which may aid in the propagation of freshwater mussels. However, mortality of hatchery-reared salmonids is usually very high immediately following stocking (Aprahamian et al. 2003), and most excysted glochidia do not seem to find a suitable substrate in which to continue their development (Buddensiek 1995, Hastie & Young 2003a).

**Harvesting and rearing of excysted (post-parasitic) juvenile mussels**

An alternative to the release of infected fish hosts carrying glochidia is the captive-rearing of juvenile mussels through the post-parasitic stage. This is expected to offer greater control over the survival and growth of mussels (Treasurer et al. 2006, Preston et al. 2007), but it represents a long term program that requires a committed

facility and staff, as several years will pass between infecting the fish hosts and the production of juvenile mussels for restoration.

It takes around 10 months for glochidia to develop on suitable salmonid fish hosts, but 95% of glochidia die before reaching this stage (Hastie & Young 2003a). After completing development, glochidia excyst from host tissue, fall away and must be collected, typically in plankton nets placed directly over outflow pipes (Buddensiek 1995). Juveniles can then be transferred to outgrow tanks and maintained for the next few years, until they are large enough to survive in the wild or taken into the next rearing phase. Some knowledge on the timing of excystment is advantageous to optimise the collection of mussel seed plan in the following spring (Hastie & Young 2003a). Hruska (1992) first proposed the concept of 'degree days' required to reach excystment, and concluded that a period of 15°C water temperature was required for the last few weeks. At captive breeding facilities in Wales, juveniles have excysted following an average of 2,381 degree days during the period 2005-2008 (range = 2,229 – 2619 degree days). By keeping a record of degree days, 150 μm mesh plankton nets can be placed over outflow pipes in anticipation of juvenile excystment, and the feeding regimes of host fish reduced to make it easier to harvest the post-parasitic juveniles. Post-parasitic juvenile mussels begin to pedal feed on algae and organic matter as soon as they fall from the fish host, and will therefore require suitable substrate for their initial development (Geist & Auerswald 2007). The transition from benthic to filter feeding represents a critical period for survival in captive breeding programs (Hastie & Young 2003a), as the early juvenile stages appear to be very vulnerable to disturbance and have narrow substrate requirements (Young & Williams 1983). Several factors are critical for their survival and growth, including substrate type, silt content, water quality, and an adequate supply of nutrients (Skinner et al. 2003, Geist et al. 2006). Barnhart (2006) found that occasional handling improved juvenile survival in N. American freshwater mussels, possibly due to the removal of silt and debris. Predation and competition by microfauna may also play an important role in early juvenile mortality (Zimmerman et al. 2003). Several methods have been employed in the culture of juvenile freshwater pearl mussels, including the use of outdoor mussel cages, semi-natural stream channels, salmonid hatching baskets, and recirculation systems (Figure 1.2).

*Mussel cages*

The use of mussel cages to rear excysted juvenile *M. margaritifera* in the wild was pioneered by Buddensiek (1995). Mortality amongst post parasitic juveniles was found to be around 70% during the first months (June-December), but decreased after the first winter. Only animals larger than 900 µm had a 50% chance of surviving to their second growing period, and all juveniles less than 700 µm in size died during the June-December period. Therefore initial size appeared to be a critical factor for survival of juvenile mussels. In a similar study in Scotland, Hastie & Young (2003a) reported a 3% survival rate after 12 months of cage rearing in the wild. In comparison, juveniles of *M. margaritifera* kept in similar mussel cages at a hatchery attained a 7% survival rate after 10 months. Thus, while mussel cages may offer some advantages for the culture of juvenile mussels under more natural conditions, current methods would need to be optimised and scaled up for conservation purposes. In this sense, an upwelling 'mussel-silo' cage system has recently been developed in North America to rear juvenile mussels in flowing waters with reduced risk of siltation.

*Semi-natural stream channels*

Preston et al. (2007) have recently assessed the merits of using hatchery raceways covered with gravel to serve as semi-natural stream channels for the rearing of encysted salmonids. Excysted mussels were allowed to fall in the substrate and complete their development, and analysis of gravel core samples approximately one year after the introduction of encysted hosts showed relatively high densities of juvenile mussels, up to 13,200 mussels in one cohort. This study was the first in the UK to culture and maintain large numbers of juvenile pearl mussels for restoration purposes, although similar methods have been used in the United States with other freshwater mussels (Williams et al. 1993, Beaty & Neves 2004). The advantages of this method is that it capitalizes on high encystment loads of artificially infected hosts, and allows glochidia to excyst under more controlled substrate and flow conditions. However, it is as yet unclear whether this method can be scaled up for long-term propagation, for how long should mussels be kept in stream channels, or what precautions are needed to harvest delicate juveniles from the natural substrate.

*Salmonid hatching baskets*

The use of hatching baskets represents the most widespread method of culturing freshwater pearl mussels during the early stages (Hastie & Young 2003a, Skinner et al 2003). Excysted juvenile mussels are collected in outflow mesh screens and transferred to indoor salmonid hatchery troughs fitted with hatching baskets covered with a 1 – 2 mm. layer of fine gravel (150 – 500 μm). Filtered river water upwells through each gravel basket, helping to reduce silt loads, while algae and organic matter enrich the gravel and provide nutrition for the juveniles. Post-parasitic mussels can be reared in this way for 12 – 18 months, until they are large enough to be transferred to larger facilities, or released into the wild (Hastie & Young 2003a). Survival of juvenile mussels reared by this method appears to have been high during the first few months post-excystment (Taylor 2007), but this was followed by high mortalities during the second year. As with other rearing systems, little is known about causes of juvenile mussel mortality in captivity, though predation by flatworms, mechanical damage, and silting up are thought to be important at the post-parasitic stage (Zimmerman et al. 2003, Barnhart 2006).

*Recirculation systems*

Recirculation systems offer greater control over environmental variables than typical flow-through facilities, and these have been tried successfully for culturing various species of freshwater mussels in North America (Jones & Neves 2002, Jones et al. 2004, 2005, Barnhart 2006), but not yet in *M. margaritifera*. Mussel recirculating systems typically consist of nested chambers with a downwelling flow at a rate of ca. 400 l/hr (Barnhart 2006). Substrate is required in recirculating systems for growth and survival, though this can perhaps make juvenile mussels more vulnerable to flatworm predation (Zimmerman et al. 2003). Supplemental feeding of unicellular green algae has also been found necessary (Barnhart 2006) but little is known about optimal algal diets. For example, survival in captivity of juveniles of the dromedary pearly mussel (*Dromus dromas*) was 30% after two weeks when fed the green algae *Nannochloropsis oculata* (Jones et al. 2004). Growth and survival of juvenile freshwater mussels appears to be higher in flow-through than in recirculating systems (Jones & Neves 2002), possibly due to diet imbalance. Early survival and growth are also higher when juvenile bivalves are reared on natural sediments rather than on commercial shellfish diets (Naimo et al. 2000), emphasizing that for many

species the formulation of algal diets constitutes one of the greatest challenges for captive rearing.

**Stocking of juvenile mussels**

Some attempts have been made to release glochidia directly into upstream tributaries to infect wild hosts, although there are no results available to ascertain the success of this strategy (Geist & Kuehn 2005). On the other hand, releases of cultured post-parasitic freshwater pearl mussels have not yet occurred, as these have not been cultured in sufficient numbers. The aim of the captive breeding of unionid mussels is to release individuals back into rivers at some point in the future. The success of the programme will ultimately depend, therefore, on the ability of captive-bred individuals to survive and reproduce in the natural environment, not on the success of the rearing programme itself. Yet, it is unknown if captive populations will adapt to the natural environment, and how juvenile mussels will fare compared to wild populations; this is an area where research is urgently needed (Hoftyzer et al 2008).

## CONCLUDING REMARKS

As with other unionid mussels, the conservation of *M. margaritifera* is problematic and exacerbated by the continuation of many practices that actively contribute to their decline (Strayer 2008). The problems of silt pollution, unsympathetic riparian management, habitat fragmentation, and declining host populations need to be addressed whilst there are still sufficient numbers of reproductively viable adult mussels. In common with other freshwater mussels (Berg et al. 2007, Zanatta & Murphy 2007, Elderkin et al 2007), *M. margaritifera* shows a significant degree of population structuring (Machordom et al. 2003), even at small spatial scales (Geist & Kuehn 2005, Bouza et al. 2007). Areas colonized by *M. margaritifera* since the last glacial maxima display high genetic diversity (Geist & Kuehn 2008; Geist et al 2009), and this may be indicative of locally adapted populations, as seen in their salmonid hosts (Garcia de Leaniz et al. 2007), and should be taken into account when developing *ex-situ* conservation programs for the species (Geist & Kuehn 2005). For example, translocations of mussels between watersheds, or introduction of artificially-reared individuals, may result in gene introgression and the break

down of local adaptations, further compromising the conservation of depleted populations. Given what has been learned over the last few decades about the genetic risks of fish stocking (reviewed in Cross et al. 2007), the artificial propagation of freshwater mussels should take into account the genetic variation, effective population size, and number and extent of neighbouring mussel conservation units. It can be argued that until the situation in rivers improves, the conservation of this species will depend on captive breeding. There may simply be too few individuals to maintain self-sustaining populations, particularly in the face of sudden pollution events, massive floods, or other catastrophes. But it can also be argued that unless the underlying threats facing the species are also addressed, captive breeding alone is unlikely to save endangered freshwater mussels from extinction. Indeed, relying on captive breeding alone is dangerous and is what Meffe (1992) termed 'techno-arrogance' and 'half-way technologies', i.e. when resources are simply diverted from habitat protection to artificial propagation, and technology is used for treating the symptoms rather than the causes of decline. Captive breeding cannot be a substitute for habitat restoration (Christian & Harris 2008), and single-species approaches are unlikely to work with pearl mussels, as these can conflict with the conservation of other species (see Geist & Kuehn 2008). Instead, success is most likely to come from multi-faceted projects which take a holistic, integral approach to conservation and rely on four underlying principles: (1) legal protection and policing, (2) public awareness, (3) habitat restoration and (4) artificial breeding.

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Table 1.1 Geographic variation in the putative salmonid hosts of the freshwater pearl mussel, *Margaritifera margaritifera*.

| Country | Putative salmonid host | Reference |
|---|---|---|
| Austria | *S. trutta* | Lahnsteinera & Jagsch (2005) |
| Belgium | *S. trutta* | Araujo & Ramos (2001) |
| Czech Republic | *S. trutta* | Hruska (1999) |
| Estonia | *S. salar, S. trutta* | Geist et al. (2006) |
| Finland | *S. salar, S. trutta* | Araujo & Ramos (2001) |
| France | *S. salar, S. trutta* | Araujo & Ramos (2001) |
| Germany | *S. trutta, S. alpinus?* | Bauer (1987a), Bauer & Vogel (1987), Buddensiek (1995) |
| Great Britain | *S. salar, S. trutta, S. alpinus?* | Young & Williams (1983), Bauer (1987a), Hastie & Young (2001,2003a) |
| Ireland | *S. salar, S. trutta* | Beasley & Roberts (1999) Preston et al. (2007) |
| Latvia | *S. trutta* | Rudzite (2004) |
| Luxembourg | *S. trutta* | Araujo & Ramos (2001) |
| Norway | *S. salar, S. trutta* | Wachtler et al (2000) |
| Portugal | *S. salar, S. trutta* | Reis (2003) |
| Russia | *S. salar, S. trutta* | Ziuganov et al (1994) |
| Spain | *S. salar, S. trutta* | Alvarez-Claudio et al. (2000), Morales et al. (2004) |
| Sweden | *S. salar, S. trutta* | Erikson et al. (1998) |
| USA (northeast) | *S. salar, S. fontinalis, S. trutta?* | Cunjak & McGladdery (1991) |

**Fig. 1.1**

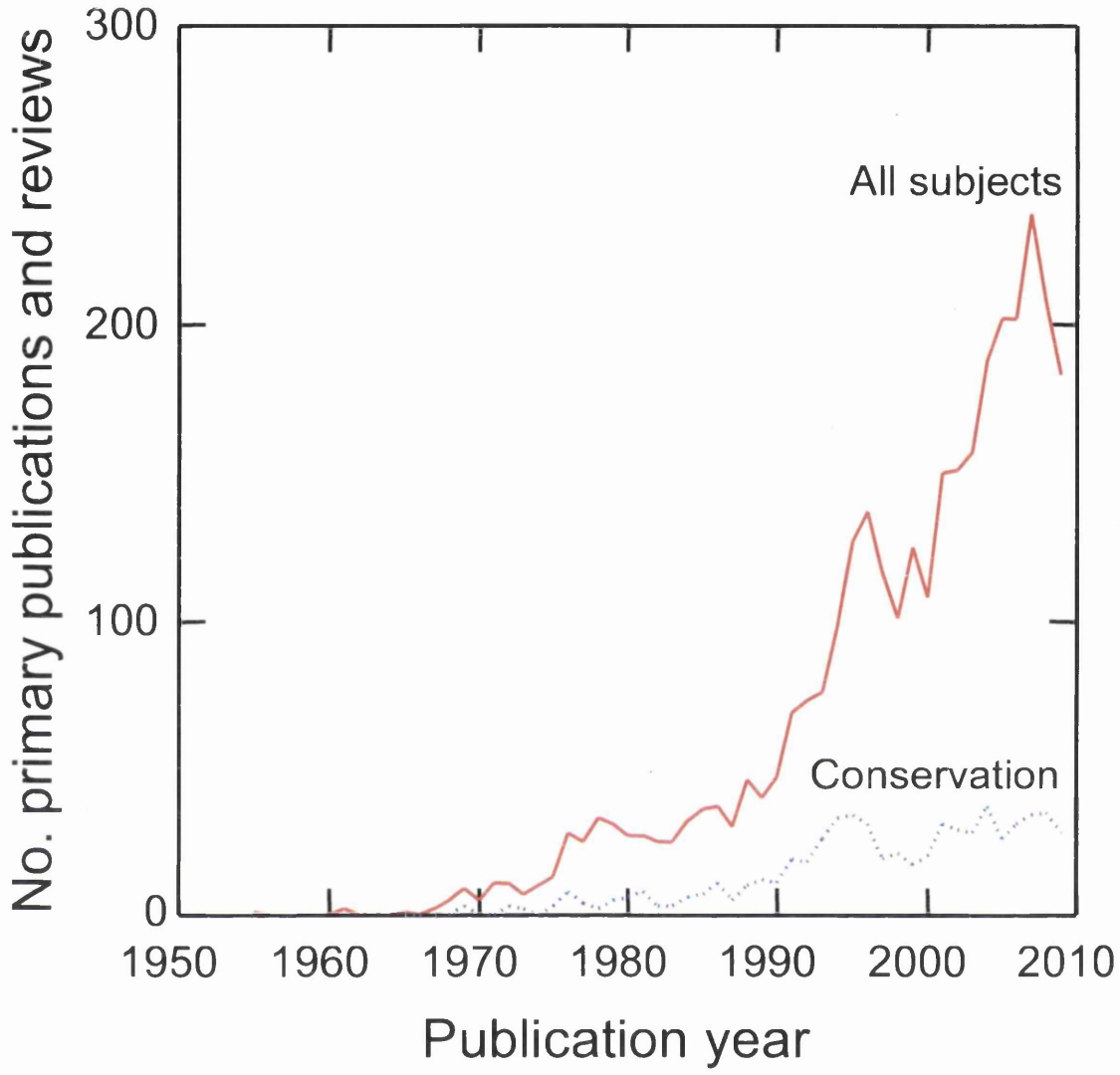


Figure 1.1. Trends in the total number of primary publications and reviews on freshwater mussels and those that deal specifically with conservation issues according to ISI Web of Science. While research effort on freshwater mussels has grown exponentially over the last two decades, relatively little of it has been directed towards addressing their conservation needs, despite the fact that freshwater mussels are becoming increasingly imperilled.

**Fig. 1.2**

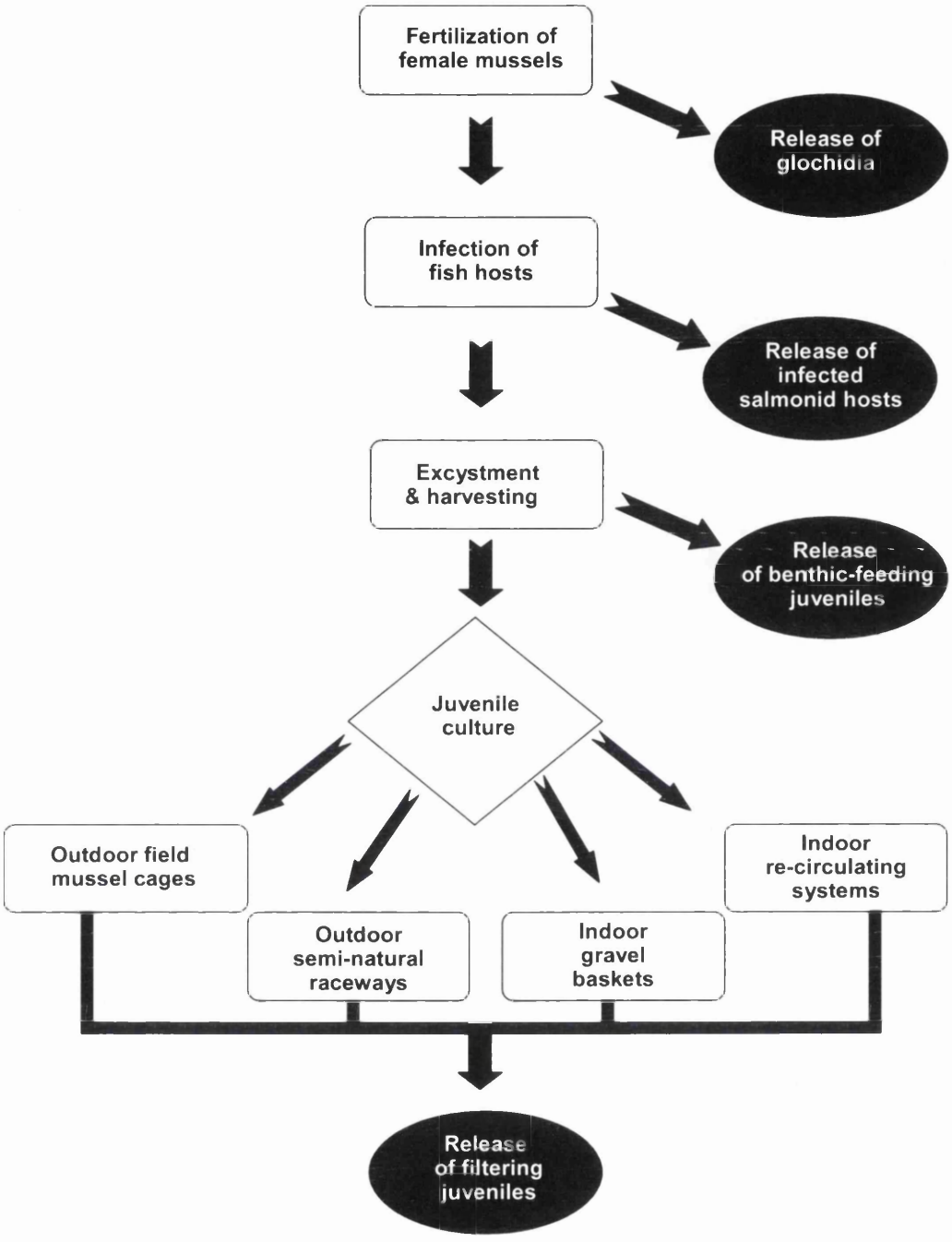


Figure 1.2. *Ex situ* conservation strategies for the propagation of the freshwater pearl mussel *Margaritifera margaritifera*.

# Chapter II.

## *In situ* conservation of the freshwater pearl mussel *Margaritifera margaritifera*

Thomas, G.R., Taylor, J., Garcia de Leaniz, C. *In situ* conservation of the freshwater pearl mussel *Margaritifera margaritifera*. (in prep.)

# Chapter II.

## *In-situ* conservation of the freshwater pearl mussel *Margaritifera margaritifera*

**ABSTRACT**

Pollution, eutrophication, habitat loss and collapse of fish hosts have all played a role in the decline of freshwater mussels worldwide. *In-situ* conservation could help protect and restore declining mussel populations, but its benefits will depend critically on addressing current anthropogenic impacts of critical mussel habitats, as well as on preventing or mitigating against future habitat losses. In this context, restoration of river connectivity, reduction of silt loads, and improvements in water quality are likely to yield the best results. *Ex situ* conservation will never be a substitute for *in situ* conservation, at best "buying time" whilst the habitat is restored, and as such should not be implemented in isolation.

**Keywords**: Freshwater pearl mussel, *Margaritifera margaritifera*, habitat, river connectivity, *in-situ* conservation

## INTRODUCTION

Freshwater mussels are considered flagship or 'umbrella' species (Bogan 2008) and play a key role in the recycling of nutrients by filtering phytoplankton, bacteria and particulate organic matter and releasing nutrients back into the river (Vaughn & Hakenkamp 2001). They also filter large volumes of water (Ziuganov et al. 1994; Mohlenberg et al. 2007), which can significantly reduce suspended sediment loads and improve water clarity (Cosgrove & Harvey 2005). Their decline can therefore impact on whole ecosystem processes (e.g. Nichols & Garling 2000; Howard & Cuffey 2006). Clean river water is an essential requirement for many aquatic organisms and the conservation of freshwater mussels can therefore have a positive effect on entire freshwater ecosystems (Skinner et al. 2003). Like all freshwater pearl mussels, the larvae of *M. margaritifera* (glochidia) are obligate gill parasites of fish, where they encyst and develop for several months before they drop off into a suitable substrate (Hastie & Young 2003a). Since healthy fish hosts are needed for their development, it has been argued that the presence of freshwater pearl mussels is therefore a good indicator of fish host populations, and in general of river integrity (Hastie & Young 2003b). In addition, the filtering behaviour and long life span of many freshwater mussels make them good bioindicators for examining the effects of climate (Hastie et al. 2003; Schöne et al. 2004) and anthropomorphic change (Brown et al. 2005).

Conservation efforts have often been hampered by limited knowledge of a species' ecological requirements, which in the case of freshwater mussels is still fragmentary (Bauer 2000; Geist 2010). Although causes of decline are numerous, and vary between populations, illegal pearl fishing, water pollution by organophosphates and other pesticides, eutrophication, habitat loss and collapse of host fish populations appear to have been particularly important and common to many areas (Young & Williams 1983; Vaughn & Taylor 1999; Cosgrove et al. 2000; Morales et al. 2004; Hastie 2006). Lack of juvenile recruitment for several decades has resulted in the overrepresentation of older mussels in many populations, and this is often one of the first and clearer signs of endangerment (Araujo & Ramos 2001; Skinner et al. 2003; but see Österling et al. 2008).

The continuing decline of many freshwater mussel species has resulted in a recent focus on their restoration and conservation in Europe (Buddensiek 1995; Beasley & Roberts 1999; Hastie & Young 2003a; Preston et al. 2007) and elsewhere (Strayer et al 2004; Barnhart 2006). In some cases entire *M. margaritifera* populations have been taken into captivity in an attempt to safeguard critical populations; the aim is to establish living gene banks for future re-stocking (Taylor 2007). Despite this recent attention, there are still large gaps in our understanding of critical stages in these animals' life history, and the relative merits of different conservation strategies. Here we critically review various strategies for the *in situ* conservation of the freshwater pearl mussel *Margaritifera margaritifera*, and draw parallels with other freshwater mussel species. We examine the main gaps in knowledge, and indicate those areas in most need of research. Our objectives are to illustrate the range of options available for *in situ* conservation of freshwater mussels, and to consider the relative merits and limitations of various restoration strategies.

## STRATEGIES FOR *IN SITU* CONSERVATION

Efforts to conserve *M. margaritifiera in situ* have stressed the need for restoring critical habitats, improving water quality (particularly by reducing silt loads), restoring river connectivity, and maintaining minimum flows (Beasley & Roberts 1999; Cosgrove & Hastie 2001; Poole & Downing 2004). In addition, adult mussels have also been translocated (both within and among watersheds) in an attempt to aid natural dispersal (Bauer 1988).

### Protection and restoration of mussel habitats – the role of freshwater reserves

The abundance of *M. margaritifera* and other margaritiferid mussels tends to be positively associated with broadleaf and mixed riparian woodland, and negatively associated with emergent reed beds and sedges (Hastie et al. 2000; Stone et al. 2004). Management of mussel habitats for conservation should therefore include strict protection of riparian buffer zones, as highest mussel densities tend to be found in shaded channels (Gittings et al. 1998). Scandinavian populations of *M. margaritifera* tend to be found in deeper waters than more southerly populations, and shade does not

probably have such an effect on mussel distribution at low temperatures. Vegetation clearance has a negative impact on freshwater mussel populations and should be avoided (Poole & Downing 2004; Brainwood et al. 2006).

Agriculture, forestry, and road management can introduce vast quantities of fine silt into rivers, which can persist many miles downstream (Wahlstrom 2006). Silt is potentially lethal for freshwater mussels and constitutes a critical factor in the survival of post-parasitic juveniles (Buddensiek et al. 1993; Weber 2005). Silt impacts on mussels by clogging up their inhalant siphons and by reducing oxygen exchange in the substrate interstitial zone (Buddensiek 1995; Beasley & Roberts 1999; Moorkens 2000).

Freshwater reserves for mussels should therefore include restoration of gravel beds, and contemplate measures designed to reduce silt loads (Cosgrove et al. 2000). Oligotrophic upland streams are particularly important for conservation as they represent important habitats for *M. margaritifera* (Geist & Kuehn 2008). Simple changes in land management, such as control of overgrazing or the establishing of riparian buffer strips, can significantly reduce pollutants and sediments from entering rivers (Roni et al. 2002; Owens et al. 2005), and these measures can greatly benefit juvenile mussels (Sparks 1995), which are particularly sensitive to poor water quality (Young 2005) and can only survive in well-oxygenated substrates (Buddensiek et al. 1993).

The influence of water velocity and river depth on the distribution of juvenile and adult *M. margaritifera* is poorly understood (Skinner et al. 2003), and this constitutes an important limitation for management of mussel habitats (see review by Strayer 2008). For example, water depth is thought to be a critical factor for the survival of freshwater mussels, as shallow waters may dry out in summer or freeze in winter, but whether mussels adjust their depth seasonally is not clear. In the British Isles adult *M. margaritifera* are found preferentially in waters 0.2-0.4 m deep and with water velocities within the range 0.25-0.75$^{ms-1}$ (Gittings et al. 1998; Hastie et al. 2000), but there appears to be considerable variation between sites. Thus, adult *M. margaritifera* have been observed at depths of 3 m in some Scottish rivers (Hastie et al. 2000), whilst on the island of Shetland both adult and juvenile *M. margaritifera* are found in small springs and trickles of water less than 10 cm deep (Cosgrove & Harvey 2005). In contrast, in

Finland adult *M. margaritifera* are found predominantly in waters between 1 and 3 m deep (Valovirta 1995), presumably to avoid freezing in winter (Hendelberg 1961). For other freshwater mussels (including *M. laevis* and *M. falcata*), optimum depth and flow velocities are within the range 0.2-0.6 m and 0.23-0.30$^{ms-1}$, respectively (Vannote & Minshall 1982; Stone et al. 2004).

Low water velocities allow algal mats to form, and silt and detritus to accumulate, thereby reducing the mixing of interstitial water, lowering oxygen levels and increasing temperature (Layzer & Madison 1995; Box & Mossa 1999; Skinner et al. 2003). These can all impact on both juvenile and adult mussels (Geist & Auerswald 2007). Moderate flooding may have a beneficial effect by removing silt accumulated over the course of the summer (when flow rates are at their lowest), but severe flooding can damage mussel populations by physically removing adults and altering suitable gravel beds (Hastie et al. 2001). For juveniles in particular, even minor hydrological changes can have a significant impact on survival (Bauer 1988), which need to be taken into account when river regulation is planned. The extent to which habitat preferences of freshwater pearl mussels vary between locations or between stages of development - or are affected by sampling limitations - is not clear and in need of further research.

**Protection of mussel hosts and restoration of river connectivity**

In some areas, the decline in mussel populations appears to have mirrored the decline in abundance of salmonid hosts (Wells & Chatfield 1992; Hastie & Cosgrove 2001), suggesting that both are interrelated (but see Bauer et al. 1991; Geist et al 2006; Osterling et al 2008). For this reason, improvement of salmonid habitats is likely to be beneficial for the conservation of *M. margaritifera* in those areas where mussel habitats have been lost. Although there are few or no specific guidelines for restoring natural habitats for the freshwater pearl mussel (but see Morales et al. 2006 for a recent model), an extensive body of literature exists on salmonid habitat restoration (reviewed in Beschta 1997; O'Grady et al. 1997; Roni et al. 2002), and this would constitute a good starting point for mussel habitat restoration. Salmonid enhancement programs can be tailored relatively easily to include the conservation needs of *M. margaritifera*, and such synergy would make conservation efforts more effective.

Large hydroelectric dams are often a main cause for loss of river connectivity, but low-head weirs can also hamper the movement of salmonids (Garcia de Leaniz 2008), thus depriving freshwater mussels of potential fish hosts (Watters 1996). Impoundments compromise the ecological integrity of rivers by altering natural temperature and flow regimes, as well as bedloads and sediment deposition rates (Ward et al. 1999). Not surprisingly, impoundments represent a major impact for freshwater mussels (Schöne et al. 2003; Brainwood et al. 2008), and can affect their distribution and abundance for considerable distances downstream (Vaughn & Taylor 1999; Morales et al. 2004). The construction of fish passes can restore some river connectivity by allowing the movements of migratory fish (Calles & Greenberg 2005; Jansson et al. 2007), and this can have a beneficial effect on *M. margaritifera* conservation. However, fish passes designed for adult fish will not normally allow the upstream passage of juvenile salmonid hosts, which are essential for upstream colonization of the freshwater pearl mussel. Fish passes alone will not address the problems posed by impoundments, which can only be fully reversed by the removal of artificial obstacles, many of which may be in disuse or coming near the end of their legal concession (Garcia de Leaniz 2008).

**Mussel translocations**

Attempts have been made to transfer adult mussels, both within and between watersheds (Hanstén et al. 1997; Lucey 2006). The earliest translocation efforts probably date back to the 19$^{th}$ century in Bavaria (Germany), when adult mussels were moved between watersheds in an attempt to expand the pearl fishing industry (Buddensiek 1995). Most attempts to transfer mussels appear to have failed (i.e. populations did not become established in the novel habitat), though the reasons for this are not clear (Scherf 1980; Valovirta 1990). There are little data on the fate of translocated mussels, only their disappearance being noted (Baer & Steffens 1987).

Freshwater mussels are found in clumped, non-random beds (Hastie et al. 2000), and it is possible that lack of recruitment in small populations may be exacerbated by an Allee effect (Petersen & Levitan 2001), caused by insufficient local densities. Some populations are at such low densities or so over-dispersed that reproduction is unlikely

to occur (Young & Williams 1983). Under these conditions, translocations and regrouping of breeding individuals could aid reproduction (Cosgrove & Hastie 2001; Cope et al. 2003; Preston et al. 2007), though removing mussels can also compromise depleted populations (Cope & Waller 1995; Waller et al. 1995). Recent mark and recapture data indicates that mature freshwater mussels are much more sensitive to handling than previously thought (Haag & Commens-Carson 2008).

## CONCLUSIONS

Freshwater mussels remain one of the world's most imperilled taxa (Strayer et al 2004), perhaps because many of the underlying stressors relate to whole catchment processes, which tend to be very difficult to address (Strayer 2008). The freshwater pearl mussel *M. margaritifera* is no exception, and many European populations display a skewed age structure, with an overrepresentation of aged adults and little or no juvenile recruitment (Araujo & Ramos 2001; Skinner et al. 2003). The first priority in the conservation of freshwater mussels should be the identification of critical stressors that contribute the most to population declines, but this has often been hampered by limited knowledge of ecological requirements at critical life stages, particularly on the most vulnerable post-parasitic juvenile stage. Thus, the microhabitat requirements of juvenile mussels is an area that deserves particular attention, as does the effect of predation on newly settled juveniles, which are still poorly understood (Hastie et al 2000; Skinner et al 2003).

The implementation of the European Water Framework Directive requires the production of management plans that consider entire river catchments, and such management plans hold considerable potential for the conservation of freshwater mussels. The post-parasitic phase of freshwater mussels tends to be the most vulnerable phase to perturbations in river processes, and gross siltation and eutrophication can have a particularly negative impact on juvenile mussels (Buddensiek et al 1993; Buddensiek 1995; Geist & Auerswald 2007). For this reason, a strict protection of riparian buffer zones designed to reduce the amount of fine silt and agricultural pollutants entering rivers represents probably one of the most effective, long-term habitat protection measures (Degerman et al 2009; Hubble et al 2010; Zhang et al 2010). As a short term strategy, simple measures such as fencing of river banks to exclude livestock have

proved useful, while riparian zones can be planted to promote medium- to long-term stabilisation of river banks (Allan 2004). However, in cases when there is already too much sediment in the substrate to allow juvenile mussel recruitment, sediment traps, gravel cleaning, and supplementary addition of coarse gravel could be beneficial (e.g. Degerman et al 2009), although the long-term benefit of such measures needs to be determined.

Restoring river connectivity to allow upstream fish migrations can benefit the conservation of various salmonid species (Garcia de Leaniz 2008 and references therein), and this should also benefit freshwater mussels that depend on salmonid hosts to complete their life cycle. River connectivity can be restored through the construction of fish passes, but also through the removal of unused obstacles, many of which may be approaching the end of their legal concession (Garcia de Leaniz 2008). Upstream colonisation by salmonids should in turn result in more juvenile fish available for glochidia encystment; although the relationship between host abundance and mussel recruitment remains obscure (Bauer et al 1991; Geist et al 2006; Osterling et al 2008).

Mussel habitat restoration has been achieved in some areas (see Geist 2010), but there are no long term data on the success of such measures. *In situ* conservation efforts should monitor the effectiveness of various methods used, and the results submitted for peer review. There are several studies detailing the restoration of salmonid habitats (reviewed in Beschta 1997; O'Grady et al. 1997; Roni et al. 2002), but few specifically aimed at freshwater mussel habitat restoration. Morales et al (2006) have proposed a model for habitat restoration for freshwater mussels, but the validity of such model has not yet been tested.

Conservation efforts have tended to focus on captive breeding alone, following improvements in artificial rearing (e.g. Preston et al 2007). However, selective forces often differ between the *in situ* and *ex situ* environments, which may result in a potential loss of fitness in juvenile mussels obtained via *ex situ* breeding (Hoftyzer et al 2008; Geist 2010). Instead, an integrative approach that combines habitat restoration with *ex-situ* breeding is likely to be most successful option (Geist 2010; Thomas et al 2010). However, no matter how much effort is directed to conservation projects, unless the

underlying threats are not first addressed at meaningful spatial scales (i.e. whole catchments), freshwater mussels will likely continue to decline.

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# Chapter III.

## Continuous monitoring of the endangered freshwater pearl mussel *Margaritifera margaritifera* (Bivalvia: Unionidae): conservation applications

Thomas GR, Robson A, Taylor J, Garcia de Leaniz C (2008) Quantifying *Margaritifera margaritifera* behaviour for live gene banking and captive breeding. *Proceedings of the International Seminar on the Rearing of Unionid Mussels*, Heinerscheid, Luxembourg ( http://www.heppi.com/presentations.htm ).

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# Chapter III.

# Continuous monitoring of the endangered freshwater pearl mussel *Margaritifera margaritifera* (Bivalvia: Unionidae): conservation applications


## ABSTRACT

The effect of sampling frequency of gape angle and exhalant pumping measurements on the ability to determine the behaviour of bivalves was examined. The endangered freshwater bivalve *Margaritifera margaritifera*, the non-endangered mussels *Mytilus edulis* and *Mytilus trossulus*, the scallop *Pecten maximus* and the cockle *Cerastoderma edule* were used as study animals. Increasing sampling interval led to an underestimation of the rate of bivalve gape adduction and abduction events detected, an overestimation of the mean duration between gape adduction and abduction events, and a misunderstanding of the form of the gape adduction and abduction events and exhalant pumping profile. Our analyses suggest minimum appropriate sampling rates for archival tags to define gape behaviour of 2, 7 and 40 Hz in *M. margaritifera*, *C. edule* and *P. maximus*, respectively, and 18 Hz to describe the metachronal wave in exhalant pumping of *M. edulis*. Careful consideration has to be given to the selection of sampling intervals when using a non-continuous method of recording behaviour. These results emphasize the importance of measuring fine-scale behaviour patterns in order to advance the understanding of bivalve behaviour. The potential loss of information associated with the choice of particular sampling intervals during measurements of single parameters, and the biases which can result from this choice, are effectively germane to all species. In this study, *Margaritifera margaritifera* displayed three distinct activity patterns, namely short duration open/close events, burrowing, and long duration filtering and/or respiration events. More generally, this study shows how the use of novel sensor technologies can shed light on neglected aspects of freshwater mussel biology, enabling managers to optimise captive rearing and improve survival.

**Keywords**: *Margaritifera margaritifera*; freshwater mussel; valve movement; Hall-effect sensor; activity patterns.

## INTRODUCTION

Current efforts to conserve *M. margaritifera* have tended to focus on *ex situ* captive breeding, with broodstock mussels kept in salmonid hatcheries or similar conservation facilities for live gene banking (Thomas et al 2010). Broodstock condition is a critical determinant of successful reproduction in bivalves; for example, in the marine oyster *Ostrea edulis* broodstock condition has a direct impact on both the quantity of larvae produced and later larval survival (Walne 1964; Gabbot & Walker 1971). The condition of captive *M. margaritifera* broodstock will therefore be critical in determining breeding success and survival of both adult mussels and the parasitic glochidia.

Siltation is considered to be a critical factor in the survival of both juvenile and adult freshwater mussels (Hastie et al 2000), although pollution by inorganic and organic compounds such as phosphates, nitrates and heavy metals, acidification and eutrophication can all have a detrimental effect on *M. margaritifera* (Skinner et al 2003). What is currently unknown are the tolerance of adult *M. margaritifera* to siltation and pollution, and the effect of short and long term exposure on their behaviour. Standard methods of assessing an organism's response to pollutants, such as using $LC_{50}$ measurements (e.g. Augsberger et al 2003; Gooding et al 2006) are not suitable for the endangered and highly protected *M. margaritifera*. As such, non-destructive methods are required that could be used to quantify *M. margaritifera* behaviour *in situ*.

The activity patterns of bivalves other than *M. margaritifera* have been studied by measuring valve movements by means of Hall-effect sensors (Wilson *et al* 2005; Robson *et al* 2007, 2009). Hall-effect sensors can quantify the responses of bivalves to environmental variables (through high resolution measurements of valve opening and closure), such as suspended silt concentrations, eutrophication and pollutants. Evidence suggests that the valve movements of various bivalves, such as *Atrina pectinata lisckeana* (Suzuki et al 2007), *Mizuhopecten yessoensis* and *Crenomytilus grayanus* (Tyurin 1991) can be utilised as bio-monitors for unfavourable environmental

conditions. As such, bivalve behaviour can be considered to be a valid method of assessing environmental conditions (Jørgensen et al. 1988; Ropert-Coudert & Wilson 2004). Several methods exist for recording bivalve behaviour, mainly involving video photography (Maire et al 2007) and Hall-effect sensors (Wilson et al 2005; Robson et al 2007, 2009). Video or photographic methods (direct observation) have inherent limitations when recording aquatic organisms, especially in turbid conditions or with organisms that burrow into sediments (Wilson et al 2005). Additionally, Wilson et al (2005) note that the quality and interpretation of results obtained by such methods is vulnerable to observer bias. The advantage of remote sensing and of Hall-effect sensors is that behaviours can be quantified without such observer bias and without disturbing the animal.

Research on bivalve behaviour has produced insights on how organisms cope with highly fluctuating environments (e.g. Jørgensen et al. 1988). Some of the questions addressed have been aimed at providing an overall view of the behaviour of a particular bivalve species. Recording behaviour with high frequency measurements has allowed questions concerning fine-scale bivalve behavioural physiology to be addressed (e.g. Trueman 1966, Hoggarth & Trueman 1967, Wilson et al. 2005). This may involve assessment of valve gape, siphon movements (changes in aperture), filtration and pumping behaviour in relation to associated environmental parameters such as depth, light, temperature, particulate matter, food availability and predator interactions (e.g. Ropert-Coudert & Wilson 2004). Although archival tags have elucidated some remarkable animal behaviours (see e.g. Ropert-Coudert & Wilson 2004 for review), selection of the correct temporal resolution, defined by the sampling interval, is critical to defining the quantity and form of behavioural events (Boyd 1993, Ropert-Coudert & Wilson 2004). Controversy about many aspects of bivalve behaviour, such as feeding, partly results from difficulties in accurately recording high frequency measurements of bivalve filtration activity (Maire et al. 2007). Maire et al. (2007) also highlight the importance of recording short-term changes in valve gape and exhalant siphon area. Direct observation of mussel gape and exhalant siphon area (e.g. Newell et al. 2001, Maire et al. 2007) has the advantage of being simple to perform; however, it does not lend itself to situations where turbidity is high or to burrowing bivalves. In addition, the

effective resolution of visual-based systems to determine changing parameters and the frequency with which observations are conducted may profoundly affect the quality and interpretation of results (e.g. Wilson et al. 2005). The use of animal-attached remote-sensing technology, in particular Hall sensors, to measure bivalve gape (Wilson et al. 2005, Nagai et al. 2006, Robson et al. 2007) circumvents many of these problems because many measurements can be made per second and the animal may live in its normal substrate. Maire et al. (2007) proposed that images acquired at a frequency of once every 15 s were sufficient to assess filtration activity precisely in *Mytilus galloprovinciallis*, although bivalve gape has also been recorded at 5 Hz (Wilson et al. 2005), 2 Hz (Robson et al. 2007), 1 Hz (Nagai et al. 2006) and once every 5 and 10 min (Riisgård et al. 2006). However, technology now exists for reliably measuring gape angle at a frequency of 32 Hz (Wilson et al. 2008). Despite its endangered status, little is known about the behaviour of the endangered freshwater bivalve *Margaritifera margaritifera* or about how to measure its wellbeing in captivity (but see Trueman 1966). We suggest that archival tag technology (Cooke et al. 2004, Ropert-Coudert & Wilson 2004), such as that used by Wilson et al. (2005) on blue mussels *Mytilus edulis*, could change this by allowing identification of normal and stressed behaviour (Robson et al. 2007). Despite the growing use of *ex situ* techniques for *M. margaritifera* conservation (Geist 2010; Thomas et al 2010), very little is known about the activity and behaviour of adult *M. margaritifera*, especially those maintained in captivity (Trueman 1966; Hoggarth & Trueman 1967; Robson et al 2009). In this study we report on the adaptation of existing technology to the study of *M. margaritifera* adults maintained in typical *ex situ* conditions. The objectives of this study are to determine if attaching such sensors to such endangered bivalves leads to post-tagging mortalities; and to identify normal and stressed behaviour without observer bias.

## METHODS AND MATERIALS

### Collection and maintenance of bivalves

All research detailed below was conducted in accordance with institutional, national and international guidelines relating to the use of bivalves in research. *Margaritifera margaritifera* used in experiments were held at the Environment Agency Wales, Cynrig Hatchery, Brecon, Wales. *Pecten maximus* were collected from the Bay of Brest, France, and transferred to a flow-through aquarium system within 2 h. Intertidal *Mytilus edulis* and *Cerastoderma edule* were collected from Swansea Bay and the Gower coast, Wales, UK, respectively, and *M. trossulus* from the coastline outside the Pacific Biological Station, Vancouver Island, Canada, at low tide and transferred to a flow-through aquarium system within 2 h.

### Experimental design

To make relative valve gape measurements in mm between bivalves of different lengths, we used methods developed by Wilson et al. (2005) and modified by Robson et al. (2007) to quantify gape angle in mussels *Margaritifera margaritifera*, *Mytilus edulis*, *M. trossulus*, the scallop *Pecten maximus* and the cockle *Cerastoderma edule.* However, neither Wilson et al. (2005) or Robson et al. (2007) calibrated all possible gape angles with sensor output and extrapolated bivalve gape calibration curves beyond known limits. Some gape data >5° were thus probably overestimated. The valve gape calibration dilemma was avoided in the present study by killing the bivalves or using a muscle relaxant on them after experiments, and calibrating Hall sensor output in mV to gape (°) over all gape angles (but see Nagai et al. 2006 who used the Hall sensor to measure bivalve gape without the need for calibration). Calibration is recommended to ensure best possible accuracy in valve gape measurements. Briefly, quantifying bivalve gape involved using a Hall sensor (a transducer for magnetic field strength) attached to one shell valve reacting to a magnet attached to the other shell valve. Variance in gaping extent produced a corresponding variance in the magnetic field strength perceived by the Hall sensor (cf. Wilson et al. 2002). This was recorded by an archival tag. Since Hall sensor output is proportional to magnetic field strength and angle of impingement, the transducer output must be calibrated by comparing shell gape angle with sensor output

over a wide variety of angles. A muscle relaxant (500 ppm buffered tricaine methanesulfonate, MS-222) (Lellis et al. 2000) was used on the endangered freshwater pearl mussels *Margaritifera margaritifera* (note *M. margaritifera* were not killed) to allow calibration of all possible gape angles with sensor output. The adductor muscle(s) of *Mytilus edulis*, *M. trossulus*, *Pecten maximus* and *Cerastoderma edule* were simply severed with a knife and bivalves were immediately calibrated for gape over all possible gape angles (~5 min per bivalve). Subsequently, data of sensor output versus gape angle were curve-fitted (for details see Wilson et al. 2002, 2005, Wilson & Liebsch 2003, Robson et al. 2007). The curve-fit could then be used to determine any gape angle by converting the transducer output accordingly. One type of archival logger used was a 13-channel JUV-Log equipped with 12 Hall sensors (Honeywell, SS59E) and 1 temperature transducer. Two other archival loggers used were 7-channel JUV-Logs equipped with 4 Hall sensors (Honeywell, SS59E) and also recorded light (lux), pressure (depth) and temperature (°C). Two further 13-channel loggers had Hall sensors linked to the logger (IMASEN, Driesen and Kern GmbH) and also recorded light, pressure and temperature. The 13- and 7-channel JUV archival loggers were powered by four 1.2 V 10 Ah NiMH D cells and the IMASEN loggers by two 3.6 V 1/2 AA lithium batteries. Each had a 1 Gb flash random access memory and could be set to record at intervals up to a maximum frequency of 2, 12 and 30 Hz, respectively. The IMASEN and JUV-Log archival loggers had 16 and 22 bit resolution, respectively, both recording gape angle at better than 0.01°. The magnets used were 5 × 5 × 2 mm neodymium boron magnets. Magnets and Hall sensors were glued to *Margaritifera margaritifera* and *Pecten maximus* using 5-minute epoxy adhesive (X003, Atlas Polymers) and Araldite® 90 Seconds (Huntsman Advanced Materials), respectively. The other bivalves kept in saltwater aquaria during experiments had their systems attached using aquarium sealant (Geocel®), and the bivalves kept in intertidal environments had systems attached using high strength epoxy adhesive (Power-Fast®+, Powers Fasteners). *M. margaritifera* had been in freshwater pumped from a local river for months before experiments began. *Mytilus edulis* and *Cerastoderma edule* were placed in an aerated flow-through aquarium system containing edible particulate matter-laden seawater from Swansea Bay for at least 1 mo before being used in aquarium experiments. *P. maximus* were placed in

an aerated flowthrough aquarium system containing edible particulate matter-laden seawater from the Bay of Brest for at least a 24 h before being used in aquarium experiments. Equipped *M. edulis* and *M. trossulus* used in intertidal experiments were returned to the intertidal within 24 h of initial collection.

**Bivalve pumping**

Lengths of PVC tubing (10 mm diameter, 1.5 mm wall thickness and lengths of 300 and 25 mm) were glued together at right angles using high strength epoxy adhesive (Fig.3.1). A Hall sensor was attached (using aquarium sealant) to the outside of the 300 mm long PVC tube, 60 mm below the 25 mm length of tubing (Fig. 3.1). A vane 60.5 mm long, 18 mm wide and 0.05 mm thick, made of translucent green Silastic® (Dow Corning) or transparent polyethylene, had one end attached to the ~25 mm long PVC tubing using aquarium sealant (Fig. 3.1). A 0.1 g (in air) neodymium boron magnet was attached at the free end of the vane using aquarium sealant so that the magnet and Hall sensor were aligned (Fig. 3.1). Pumping sensors were kept in a fixed position in mussel tanks using PVC clamps. The study mussel was then placed in relation to the vane so that the water exhaled (from the top 10 mm of the inhalant siphon and whole of the exhalant siphon) caused the vane to move, bringing the magnet closer to the Hall sensor, thus causing a change in magnetic field intensity perceived by the transducer (in a manner similar to that used for determining gape angle, see above). It was imperative to keep the Hall sensors and magnets from the gape and pumping sensors sufficiently far apart so they did not interact. In preliminary pumping experiments *Mytilus edulis* used their foot to move the translucent green Silastic® vane out of the path of their exhalant water current and stuck it to the outside of their shell. This did not occur over 12 months of continuous pumping experiments using transparent polyethylene as the pumping sensor vane. Thus, transparent polyethylene was used as the pumping sensor vane in the present study. Sampling frequencies of 2 and 30 Hz were used to record *M. edulis* pumping. The new method for measuring pumping could not be used in strong currents because of the high sensitivity of the sensor. We did not attempt to calibrate the fine temporal and sensor resolution exhalant pumping data because of complications our system could not easily account for. Complications include: (1) *Mytilus edulis* exhalant

pumping can occur from the top of the inhalant siphon in addition to the exhalant siphon— there is no defined barrier to exhalant pumping from the top of the inhalant siphon, and it may not be assumed that inhalant pumping occurs throughout the whole of the inhalant siphon area (and clearly not when exhalant pumping occurs from the top of the inhalant siphon) (2) Both changes in mussel siphon area and siphon orientation relative to the pumping sensor will change the force per unit area exerted on the pumping sensor. (3) *M. edulis* valve adduction events further complicate the measurement of exhalant pumping because maximum recorded exhalant pumping in this study was not produced by pumping (cilia beat) but by valve adduction (thus it is important to also measure valve gape in tandem with exhalant pumping at high temporal and sensor resolution so these two types of currents can be separated).

**Experiments**

Examples of bivalve gape behaviour at various sampling frequencies in the present study were obtained from *Margaritifera margaritifera*, ($n$ = 6, mean length 107.8 ± 7.1mm SD), 79 *Mytilus edulis* (gape and pumping in 48 *M. edulis)*, 10 *Cerastoderma edule* and 7 *Pecten maximus* in laboratory aquaria as well as 52 *Mytilus* spp. in the intertidal zone (Atlantic and Pacific). Bivalves in their natural environments fed on natural seston and bivalves in aquarium experiments fed on seston pumped from their natural environment. Experiments with bivalves took place from December 2006 to April 2008.

## RESULTS

### Impact of Hall sensors

No mortalities of the endangered *M. margaritifera* were recorded during the five months of the study period, both sensors and magnets were later removed from the mussels, and none of the experimental animals died in the six months following sensor removal.

### Bivalve gape

In preliminary investigations with live bivalves we made sure that our best-fit gape angle calibration curves for live animals were similar to those for sacrificed individuals. As an example, we used ANCOVA to compare 2 methods of gape calibration repeated in triplicate on one *Mytilus edulis*: (1) gape calibration on the live mussel and (2) gape calibration after the posterior adductor muscle was severed. Gape calibration method was the fixed factor and gape angle was the continuous variable. There was no significant effect of calibration method in the model ($F_{1,39}$ = 0.148, $p$ = 0.702). Calibration of maximum gape angle was not possible in live bivalves; the majority of any error in gape calibration curves was probably caused by human error (all best-fit calibration curves had $r^2 > 0.98$). All major *Mytilus edulis* gape movements recorded at 2 Hz (0.5 s) followed the same general pattern as those recorded at 30 Hz (see Fig. 3.2). The rate of reduction in valve gape angle (adduction) was faster than the subsequent increase in gape angle (abduction), the latter having a roughly logarithmic form, in *M. edulis* (Figs. 3.2 & 3.3), *M. trossulus* (Fig. 3.4) and *Margaritifera margaritifera* (Fig. 3.5), with the rate decreasing near the endpoints of both adduction and abduction events. During recording of gape at 2 Hz in the smaller and faster-moving *Cerastoderma edule*, the rate of valve abduction did not always decrease near the endpoints of every abduction event (Fig. 3.6). Close inspection of *C. edule* gape data (Fig. 3.6) revealed that all valve adduction events occurred at a faster rate than the subsequent abduction event. Reduction in gape sampling frequency was associated with a progressive change in the shape of the gape angle versus time graph in both non-burrowing and burrowing bivalves in saltwater aquaria (Figs. 3.2 & 3.6, respectively) and in wild Pacific intertidal marine bivalves (Fig. 3.4). Reducing sampling frequency below 2 Hz (intervals of 0.5 s) made valve movements appear to be faster than they actually were (Figs. 3.2, 3.4 – 3.6).

Accurate assessment of short-term changes in valve gape was only possible recording *Margaritifera margaritifera* gape at intervals of ≤0.5 s (Fig. 3.5). Increasing the sampling interval of gape data from 0.5 to 10 s resulted in the loss of some complete valve adduction and subsequent abduction events (e.g. Fig. 3.5). Visual observation of *M. margaritifera* burrowing behaviour backed up by recording gape at 0.5 s intervals (e.g. Fig. 3.5) highlighted the importance of valve movement for burrowing into sediment. In one example, sampling at 1 to 5 s intervals, 45 valve adduction and subsequent abduction events over 1 h of *Margaritifera margaritifera* burrowing activity were plotted as a plateau with downward spikes. Increasing the sampling interval to ≥10 s concentrated some adjacent gape adduction and abduction events, with only 10 valve adduction and subsequent abduction events detected when sampling at 60 s intervals (Fig. 3.7). Over 1 h of burrowing activity, mean, median and minimum *M. margaritifera* gape angle increased as the sampling interval increased from 0.5 to 60 s (Table 3.1). Increasing the sampling interval from 0.5 to 60 s caused the interquartile range of *M. margaritifera* gape data to decrease by 0.59° and caused median gape to increase by 0.31° (Table 3.1). Maximum gape of *M. margaritifera* and *Pecten maxiumus* decreased by 0.1° and 4.72°, respectively, when the sampling interval was reduced from 0.5 to 60 s (Table 3.1). Over 1 h there was no change in mean gape but there was a reduction in maximum gape angle of *M. margaritifera* and *Mytilus edulis* when the sampling interval was increased from 0.5 to 5 s (Table 3.1). Also over 1 h there was no change in mean gape but there was a reduction in maximum gape angle of *Pecten maximus* when sampling frequency was decreased from 12 Hz (sampling interval of ~0.083 s) to once every 0.5 s (Table 3.1). However, over 1 h of *Cerastoderma edule* gape data, there was a change in mean gape and a decrease in maximum gape angle when the sampling interval increased from 0.5 to 5 s.

**Pumping**

A reduction in sampling frequency of bivalve pumping behaviour was associated with a loss in definition of short-term changes in exhalant pumping (Fig. 3.8). At fine scales (2 Hz), *Mytilus edulis* gape was well defined, while at the same frequency, pumping was apparently rarely constant and did not appear to be fully elucidated (e.g. Fig. 3.8).

Mussel pumping recorded at 30 Hz revealed apparent and variable noise (a metachronal wave) in the pumping data of all animals (Fig. 3.9). We determined that the metachronal wave in the pumping data was biological in origin since it was not present when the pumping sensor was used on immersed dead mussels, or when gravity-fed water flowed out of an immersed, modelled mussel exhalant siphon (made from Silastic®, Dow Corning) towards the pumping sensor.

**Measurements per event**

Recording at 2 Hz, measurements (data points) per valve adduction and subsequent abduction event were counted for 50 events from 6 *Margaritifera margaritifera* (105 ± 1.4 mm length) and 10 *Cerastoderma edule* (28.6 ± 1.9 mm length). On average, fewer measurements were made per continuous valve adduction event compared to the subsequent abduction event in both *M. margaritifera* and *C. edule* (mean numbers of measurements per adduction and abduction event were 16.0 ± 5.7 and 44.3 ± 10.9, and 4.6 ± 1.5 and 9.1 ± 3.7 in *M. margaritifera* and *C. edule*, respectively), with a minimum of 10 and 3 measurements per adduction event in *M. margaritifera* and *C. edule*, respectively. Complete *M. margaritifera* and *C. edule* valve adduction and subsequent abduction events had mean numbers of measurements per event of 54.5 ± 11.5 and 14.0 ± 4.7, respectively. Recording at 12 Hz, measurements per valve adduction and subsequent abduction event were counted for 50 events from 4 *Pecten maximus* (107.3 ± 1.7 mm length). Mean numbers of measurements per adduction and abduction event were 12.1 ± 6.8 and 789.2 ± 780.2, respectively, with a minimum of 3 measurements per adduction event. Complete *P. maximus* valve adduction and subsequent abduction events had a mean number of measurements per event of 1062.4 ± 766.1. Recording at 30 Hz, measurements per metachronal wave were counted for 50 metachronal waves from pumping data of 10 *Mytilus edulis* (69.8 ± 1.6 mm length). A mean of 30.5 ± 9.4 measurements was counted per metachronal wave, with a minimum of 17 measurements per wave.

**Behaviours of *M. margaritifera***

Distinct behaviours were identified for *M. margaritifera*, occurring over short (1 – 5 sec.; Fig. 3.10a), medium (minutes; Fig. 3.10b) and longer (hours; Fig. 3.10.c) time periods. The short and medium duration events are composed of repeated open/close events, whilst the longer events are composed of period of extended opening. Short duration single open/close events of < 5 sec. have been previously interpreted as a clearing of detritus/suspended matter from the inhalant siphons (Suzuki et al 2007) and have been termed 'vomiting'. On the other hand, multiple short duration open/close events have previously been associated with burrowing behaviour in bivalves (Suzuki et al 2007), and this was also supported by visual observations of *M. margaritifera* in the present study, particularly after mussels had been handled. Longer periods of opening (lower relative mV values) are interpreted as filtering and/or respiration behaviour (Figure 3.10) and appear common among healthy mussels, this being supported by visual observation of extended gill filaments.

## DISCUSSION

### Gape

The general patterns of *Margaritifera margaritifera* valve movements recorded at 2 Hz (e.g. Fig. 3.5) were the same as those for non-endangered *Mytlius* spp. (e.g. Figs. 3.2 – 3.4) and as previously described by Robson et al. (2007). Both the present study and the pioneering work by Trueman (1966) and Hoggarth & Trueman (1967) recorded *M. margaritifera* valve movements, although we have found no published material on the subject in the interim. We believe that bivalve valve adduction and subsequent abduction events constitute a normal part of bivalve behaviour of both endangered and non-endangered bivalves, occurring in the wild subtidal (e.g. Wilson et al. 2005) and intertidal (Fig. 3.4), simulated intertidal (Shick et al. 1986) and in laboratory aquariums (e.g. Figs. 3.2, 3.3, 3.5 & 3.6; Trueman 1966, Hoggarth & Trueman 1967, Robson et al. 2007). Adult *Cerastoderma edule* are similar in size to the critically endangered little winged pearly mussel *Pegias fabula*, which rarely exceed 35 mm in length (Bogan 2002); therefore, gape data from *C. edule* (Fig. 3.6) may be a good proxy for small endangered bivalves. *C. edule* data (Fig. 3.6) also highlight that there can be greater variability in valve movements of smaller bivalves than in larger bivalves such as *Margaritifera margaritifera* (Fig. 3.5), indicating that recording gape of small endangered bivalves at higher frequency (i.e. >2 Hz, see 'Discussion - Sampling frequency and resolution of bivalve behaviour') may be appropriate (cf. Peters 1983). Adult *Pecten maximus* are similar in size (15 cm maximum shell diameter) to another marine Pectinid, the IUCN Red Listed *Nodipecten magnificus*, which commonly approaches 20 cm in shell diameter (Waller 2007). *P. maximus* gape data highlight the rapid speed at which this scallop, and probably *N. magnificus*, can adduct. The ratios of adductor muscle(s) volume/weight to shell volume/weight in *P. maximus* will undoubtedly be lower than in *Margaritifera margaritifera,* although due to their endangered status *M. margaritifera* could not be sacrificed to quantify the ratios and may account for the rapid speed of valve adduction in *P. maximus* compared to *M. margaritifera* (see 'Discussion - Sampling frequency and resolution of bivalve behaviour').

**Pumping**

Although an accurate quantified measure of exhalant mussel pumping was not possible in the present study (see 'Materials and methods - Bivalve pumping') (cf. Ait Fdil et al. 2006), our results suggest that pumping should be measured over fine temporal scales because we found mussel pumping (and gape) to be often highly variable, even over periods as short as 1 min (cf. Robson et al. 2007). When measuring *Margaritifera margaritifera* exhalant pumping, especially in relation to gape angle, it may be beneficial to test whether an exhalant current exits from the top of the inhalant siphon as well as the exhalant siphon. *Mytilus edulis* has a mucociliary rejection pathway that functions via the inhalant siphon with pseudofaeces eliminated along the ventral side of the septum dividing the inhalant siphon from the exhalant siphon (Widdows et al. 1979, Beninger & St-Jean 1997, Beninger et al. 1999). Along with our own observations of *M. edulis* pseudofaeces strings being eliminated in an exhalant water current out of the top of the inhalant siphon (sometimes when the exhalant siphon was closed), we found it was appropriate to measure exhalant *M. edulis* pumping out of both the top of the inhalant siphon and the entire exhalant siphon.

**Biological noise**

Further research is necessary to determine the cause of the biological noise in the form of a metachronal wave of varying amplitude in *Mytilus edulis* exhalant pumping recorded at 30 Hz (e.g. Fig. 3.9). Wilson et al. (2005) reported biological noise in the gape data of bivalves (also present in our gape data) that was consistently higher in sand mussels *Astarte borealis* than *M. edulis*. Wilson et al (2005) suggested that this biological noise could be due to mussel heart beat influencing the recording equipment (cf. Curtis et al. 2000). While there is little known about the metachronal wave in mussel pumping, it may be an important parameter to measure in bivalves since the frequency of metachronal waves in pumping may vary according to biotic and abiotic factors (e.g. temperature).

**Sampling frequency and resolution of bivalve behaviour**

This study reveals the degree to which intervals between sampling affect our ability to identify bivalve gape adduction and abduction events, the degree of variability in bivalve pumping and, ultimately, how this affects the descriptive statistics of gape and pumping behaviour. One effect of increasing the sampling interval was to concatenate adjacent gape adduction and abduction events in the data record (Figures 3.2, and 3.4 – 3.7), which resulted in an increased mean duration between gape adduction and abduction events and increased minimum gape angles (Table 3.1); this is an analogous process to the effect of increasing sampling interval on the diving behaviour of seals (Boyd 1993). Another effect of increasing the sampling interval was the substantial change to the shape of bivalve gape adduction and abduction events (Figs. 3.2, 3.4 – 3.6) and pumping profiles (Fig. 3.8). Increasing the sampling interval from 0.5 to 60 s had relatively little effect on the mean gape of bivalves (Table 3.1). However, it was apparent that increasing the sampling interval from 0.5 to 5 s caused a reduction in maximum gape and thus a loss of definition in short-term changes in bivalve gape (Table 3.1). It is essential to select the correct temporal resolution defined by sampling interval in order to detect and define fine-scale behaviour patterns. If the shape of an event is described via changing values in the measured parameter, then the recording frequency should be on the order of 10 measurements per event (Ropert-Coudert & Wilson 2004). Given this, our data analysis indicates that gape should be recorded at a minimum of 2, 7 and 40 Hz in *Margaritifera margaritifera*, *Cerastoderma edule* and *Pecten maximus*, respectively, and at 18 Hz to describe the metachronal wave in exhalant pumping of *Mytilus edulis*. Where the peak values in the measured event are important, such as peaks in bivalve pumping amplitudes (Fig. 3.9) and the exact start and fastest part of valve adduction events, 10 measurements per event may not adequately describe these extremes. We note that some *P. maximus* valve adductions could not be defined (10 measurements per event) with any of the loggers used in the present study or daily diary loggers (Wilson et al. 2008). From our experience measuring bivalve pumping, we speculate that an initial sampling frequency of 30 Hz would be required to determine the appropriate sampling frequency to measure fine-scale bivalve siphon movements (changes in aperture) of *Margaritifera margaritifera*.

An inherent problem in dealing with bivalve data measured at high sampling frequency (e.g. 2 to 30 Hz) over days, weeks and months is data processing time. A computer with 8 GB RAM and a 3.4 GHz Pentium 4 processor takes ~40 min to convert 7 million gape data points (~64.8 h and ~40.5 d of data from an archival tag channel recording at 30 and 2 Hz, respectively) from only one bivalve in mV to degrees (°), using an exponential equation in the form $y = a + b \exp(-x/c)$ in Origin® version 7.5 (OriginLab). A way around this is to thin data so that curve-fits can be applied to much fewer data points. However, too few data points in the time series leads to poor resolution of behaviour which can lead to misinterpretation.

**Temporal resolution**

In the present study, with a 1 GB flash memory card and the system set to record at 30 Hz on 2 channels, recording bivalve gape and pumping simultaneously, the archival tag could record for ca. 70 d before the memory was full. Using 128 GB compact flash memory cards (Samsung) the recording times of the archival loggers could be multiplied by 128. A computer programmed interface could stop the logger just before the memory card was full, the full memory card replaced and logger restarted within 10 min. Thus, it is possible to record high temporal resolution data almost continuously.

**Implications for *M. margaritifera* conservation**

The interface between behaviour and conservation is a relatively new subject area (Caro 2007) which has the potential to improve the success of reintroduction programmes stemming from explicit consideration of organisms' behaviours (Anthony & Blumstein 2000). This is of particular importance for organisms that are subject to captive breeding, as adaptation to the captive environment can result in the expression of disadvantageous behaviours when those animals are released into the wild (Berejikian et al 2001; Kelley et al 2006).

Our method of assessing the behaviour of rare and endangered bivalves is shown to be effective and does not harm the mussels. The methods described holds the potential to monitor mussel behaviour both *in situ* and *ex situ*. For mussel populations maintained in hatcheries for captive breeding, the method described here can be used to

quantify events such as reproduction and spatting (the release of glochidia), allowing managers to better co-ordinate the captive breeding effort. *In situ* mussel responses to spates and sedimentation events could also be examined, informing the development of better guidelines for mussel habitat restoration. The high resolution measurement of valve opening and closing allows the quantification of *M. margaritifera* behaviour in response to environmental variables without adversely impacting the animals.

## CONCLUSIONS

The potential loss of information associated with the choice of particular sampling intervals during measurements of single parameters, and the biases which can result from this choice, are effectively germane to all species (cf. Boyd 1993). The analyses presented here demonstrate that careful consideration has to be given to the selection of intervals between sampling when using a non-continuous method of recording behaviour. We believe that, where possible, all behavioural events should be recorded because they are likely to vary according to biotic or abiotic factors (e.g. Wilson et al. 2005, Robson et al. 2007). The techniques and methods described can be used to identify distinct behaviours, an advancement that can be used to assist in the development of *ex situ* conservation for the endangered *M. margaritifera*. Given that the minimum appropriate sampling frequency has now been established for recording fine-scale *Margaritifera margaritifera* gape and, most probably, pumping behaviour, our ongoing research can test if the breakthrough in the ability to culture *M. margaritifera* (Preston et al. 2007) can be further improved by conditioning broodstock and providing juveniles with additional food. Archival tags such as those used in this study do not have an impact on mortality either during attachment or after sensor removal. As such, this technology can be considered suitable for use with endangered bivalves. Advances in the understanding of bivalve feeding and reproductive strategies may be gleaned by recording behaviour with high temporal and sensor resolution over a range of ecological circumstances (according to factors such as depth, light, temperature, particulate matter, food availability and predator interactions) and may aid long-term survival of endangered bivalves including freshwater pearl mussels.

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Table 3.1. Mean ± SD, median, maximum, minimum and interquartile range of gape data at different sampling intervals over 1 h from a burrowing, 100 mm long freshwater pearl mussel *Margaritifera margaritifera* in an aquarium, a 110 mm long scallop *Pecten maximus* in an aquarium, a 67 mm long *Mytilus edulis* immersed in the intertidal zone at Swansea Bay, UK, and a 28 mm long cockle *Cerastoderma edule* in an aquarium.

| Sampling interval (s) | Gape (°) Mean | Median | Max. | Min. | Interquartile range (°) |
|---|---|---|---|---|---|
| ***Margaritifera margaritifera*** | | | | | |
| 0.5 | 3.76 ± 1.34 | 3.7 | 5.9 | 0.79 | 2.35 |
| 5 | 3.76 ± 1.34 | 3.7 | 5.87 | 0.8 | 2.32 |
| 10 | 3.76 ± 1.34 | 3.7 | 5.87 | 0.8 | 2.35 |
| 15 | 3.76 ± 1.34 | 3.72 | 5.87 | 0.87 | 2.33 |
| 30 | 3.80 ± 1.31 | 3.75 | 5.87 | 0.87 | 2.39 |
| 60 | 3.82 ± 1.31 | 4.01 | 5.8 | 0.87 | 1.76 |
| ***Pecten maximus*** | | | | | |
| 0.083 | 3.31 ± 1.58 | 3.44 | 10.81 | 0.97 | 2.76 |
| 0.5 | 3.31 ± 1.58 | 3.44 | 10.69 | 0.97 | 2.76 |
| 5 | 3.30 ± 1.57 | 3.53 | 10.03 | 0.98 | 2.76 |
| 10 | 3.31 ± 1.59 | 3.39 | 10.03 | 0.98 | 2.76 |
| 15 | 3.29 ± 1.54 | 3.54 | 8.52 | 0.98 | 2.75 |
| 30 | 3.27 ± 1.56 | 3.11 | 8.52 | 0.98 | 2.74 |
| 60 | 3.25 ± 1.53 | 3.02 | 5.97 | 0.98 | 2.83 |
| ***Mytilus edulis*** | | | | | |
| 0.5 | 3.28 ± 0.98 | 3.15 | 6.27 | 0.45 | 0.41 |
| 5 | 3.28 ± 0.98 | 3.15 | 6.24 | 0.48 | 0.4 |
| 10 | 3.28 ± 0.98 | 3.15 | 6.24 | 0.56 | 0.4 |
| 15 | 3.28 ± 0.98 | 3.16 | 6.19 | 0.72 | 0.41 |
| 30 | 3.28 ± 0.97 | 3.15 | 6.19 | 0.72 | 0.41 |
| 60 | 3.29 ± 0.94 | 3.16 | 6.09 | 1.37 | 0.35 |
| ***Cerastoderma edule*** | | | | | |
| 0.5 | 4.57 ± 0.52 | 4.76 | 6.36 | 1.18 | 0.53 |
| 5 | 4.56 ± 0.54 | 4.75 | 6.18 | 1.19 | 0.54 |
| 10 | 4.57 ± 0.54 | 4.75 | 6.18 | 1.36 | 0.54 |
| 15 | 4.55 ± 0.57 | 4.75 | 5.99 | 1.19 | 0.54 |
| 30 | 4.53 ± 0.60 | 4.74 | 5.49 | 1.36 | 0.54 |
| 60 | 4.51± 0.67 | 4.75 | 5.33 | 1.36 | 0.54 |

**Fig 3.1.**

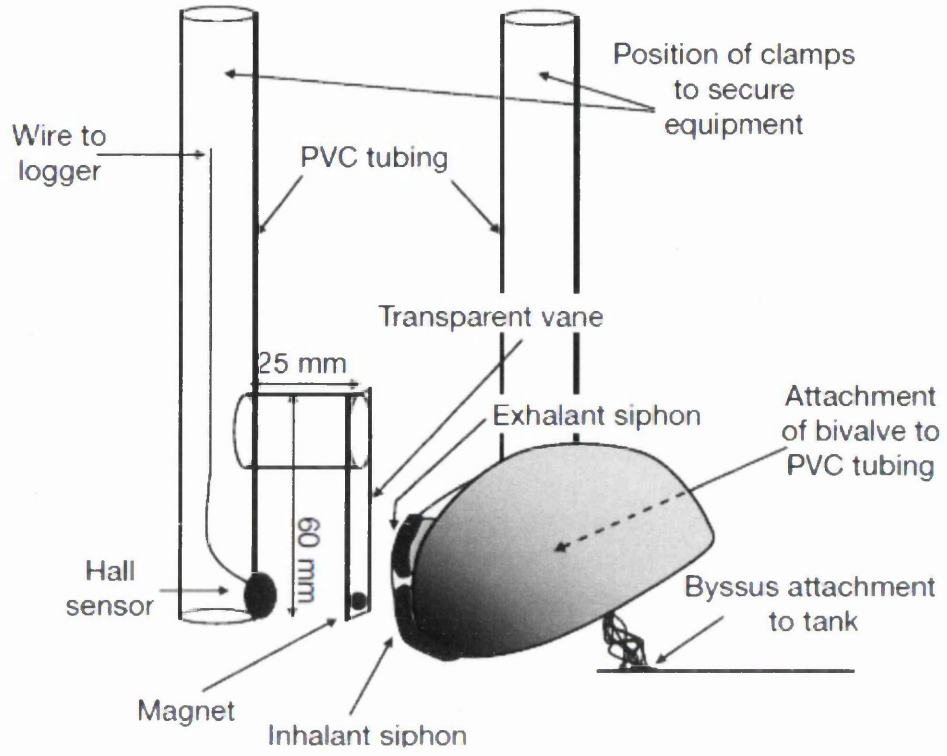


Figure 3.1. *Mytilus* spp. Schematic diagram showing the bivalve pumping sensor for measurement of the flow of water out of the top of the inhalant siphon and whole of the exhalant siphon (aperture). See Wilson et al. (2005) for a schematic diagram showing the attachment of the Hall sensor and magnet system used for determining bivalve gape angle.

**Fig 3.2.**

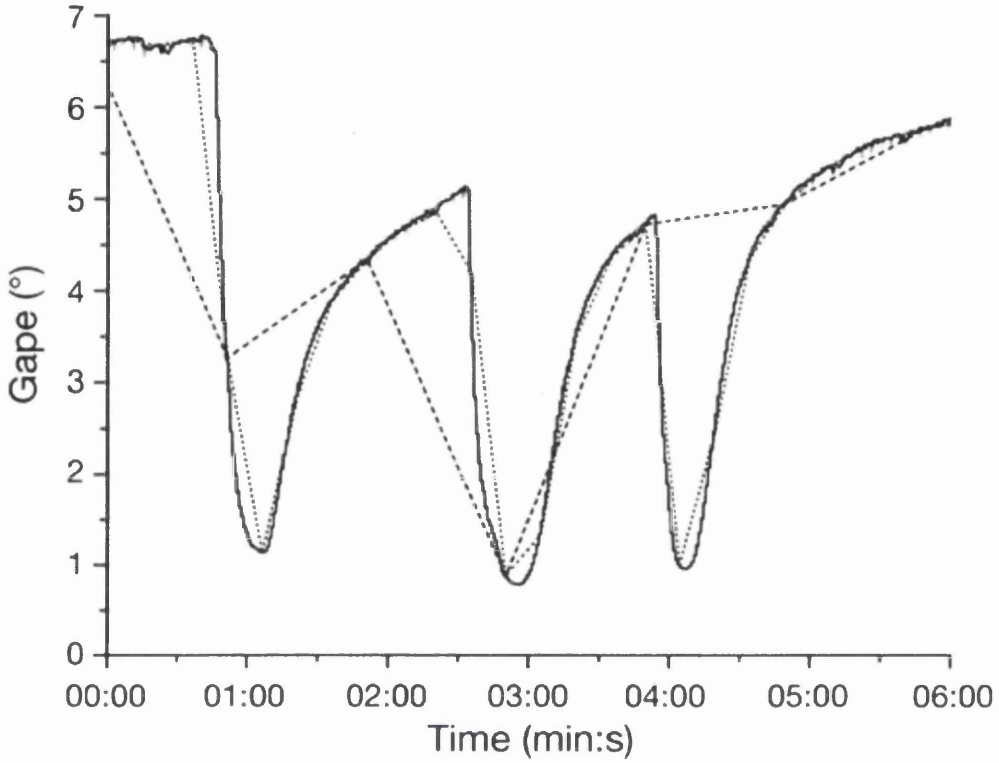


Fig.3.2. *Mytilus edulis*. Example of the effect of sampling frequency on the gape data from a 70 mm long mussel in an aquarium at Swansea University, UK. Sampling frequencies: 2 Hz (once every 0.5 s) (—), 0.067 Hz (once every 15 s) (• • •) and 0.017 Hz (once every 60 s) (---). The difference between valve gape recorded at 2 and 30 Hz is almost indistinguishable except between approximately 00:00 and 00:30 min:s

**Fig 3.3.**

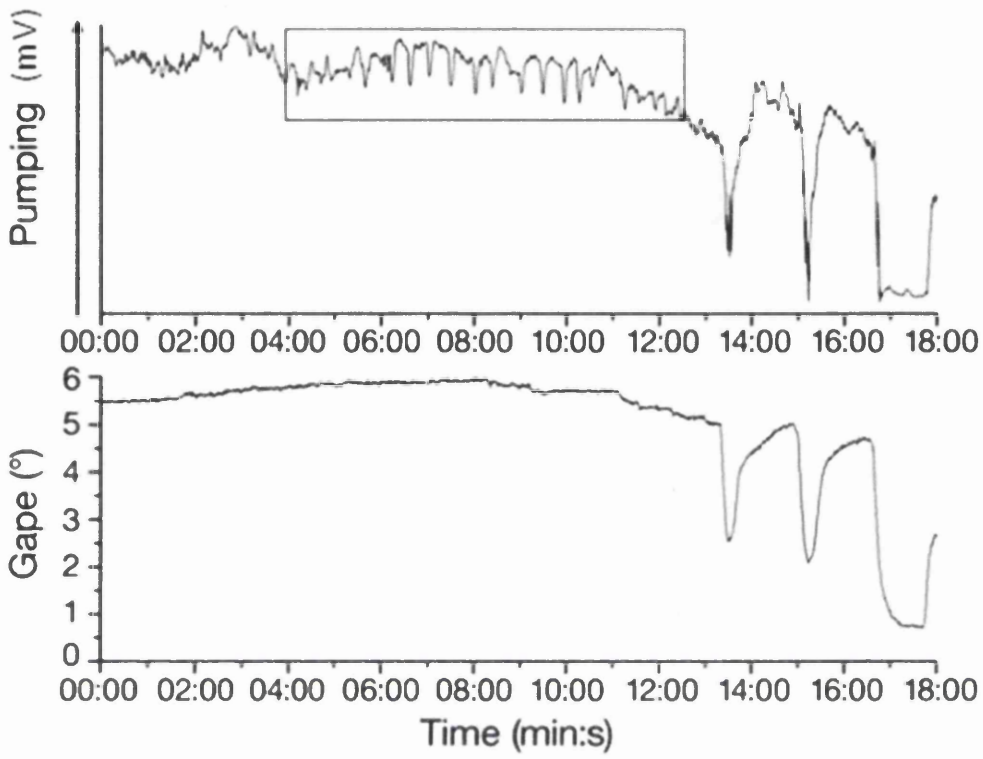


Fig. 3.3. *Mytilus edulis*. Detailed example of exhalant pumping and gape data recorded at 2 Hz from a 72 mm long mussel in a seawater aquarium at Swansea University, UK. Inset box in top panel highlights poorly defined variation in exhalant pumping.

**Fig 3.4.**

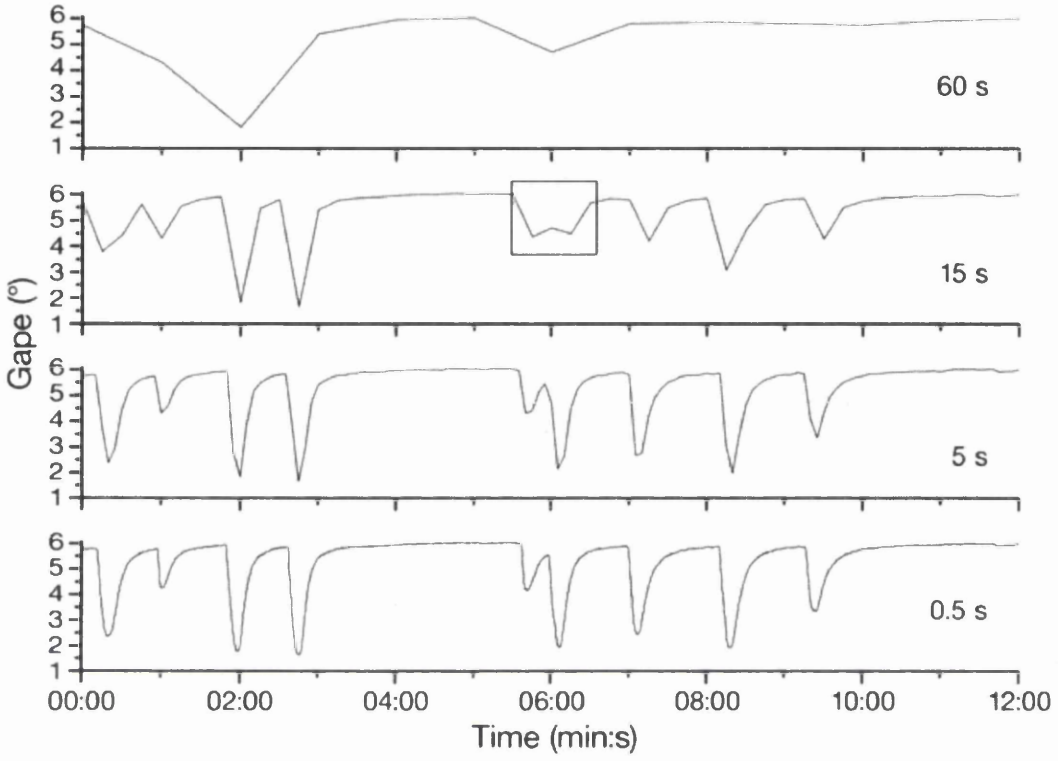


Fig. 3.4. *Mytilus trossulus*. Example of the effect of sampling frequency on gape data from a 55 mm long mussel in the Pacific intertidal zone, Vancouver Island, British Columbia, Canada. Sampling occurred once every 0.5, 5, 15 and 60 s. Box highlights the concatenation of adjacent gape adduction and abduction events in the data record that sampled once every 15 s.

**Fig 3.5.**

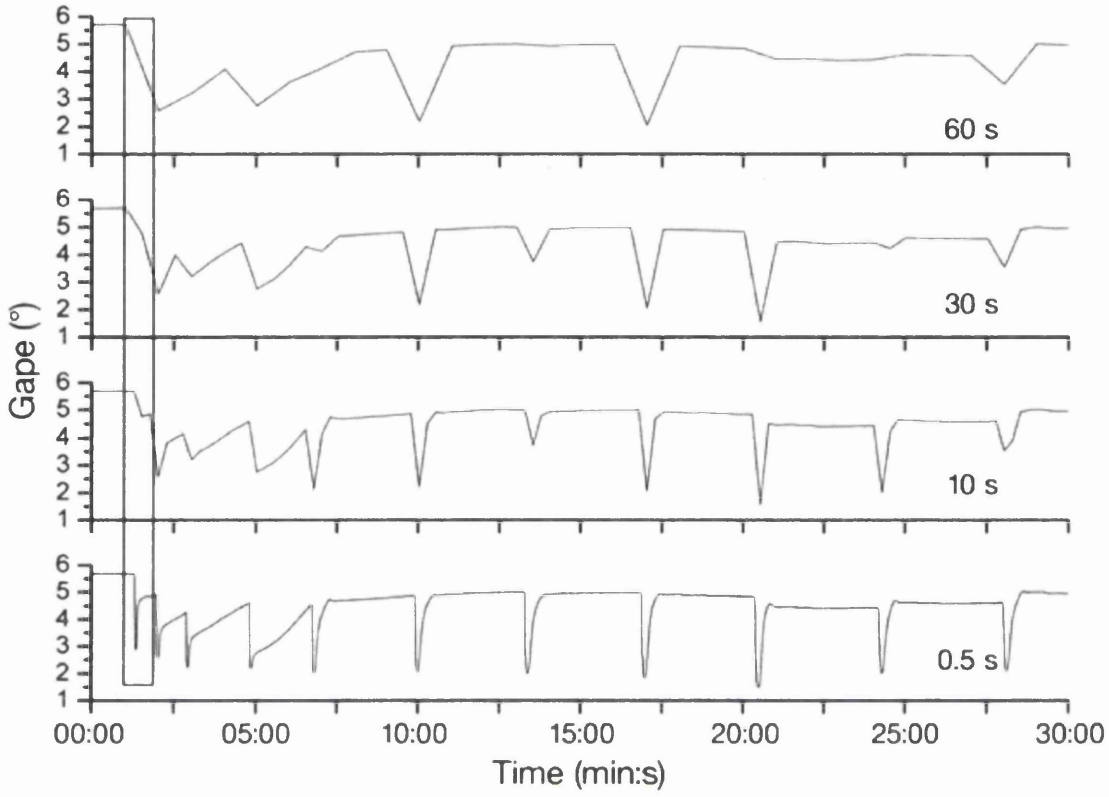


Fig. 3.5. *Margaritifera margaritifera*. Example of the effect of sampling frequency on burrowing gape data from a 100 mm long, freshwater pearl mussel in an aquarium. Sampling occurred once every 0.5, 10, 30 and 60 s. Box highlights the data loss of a valve adduction and subsequent abduction event with decreasing sampling frequency.

**Fig 3.6.**

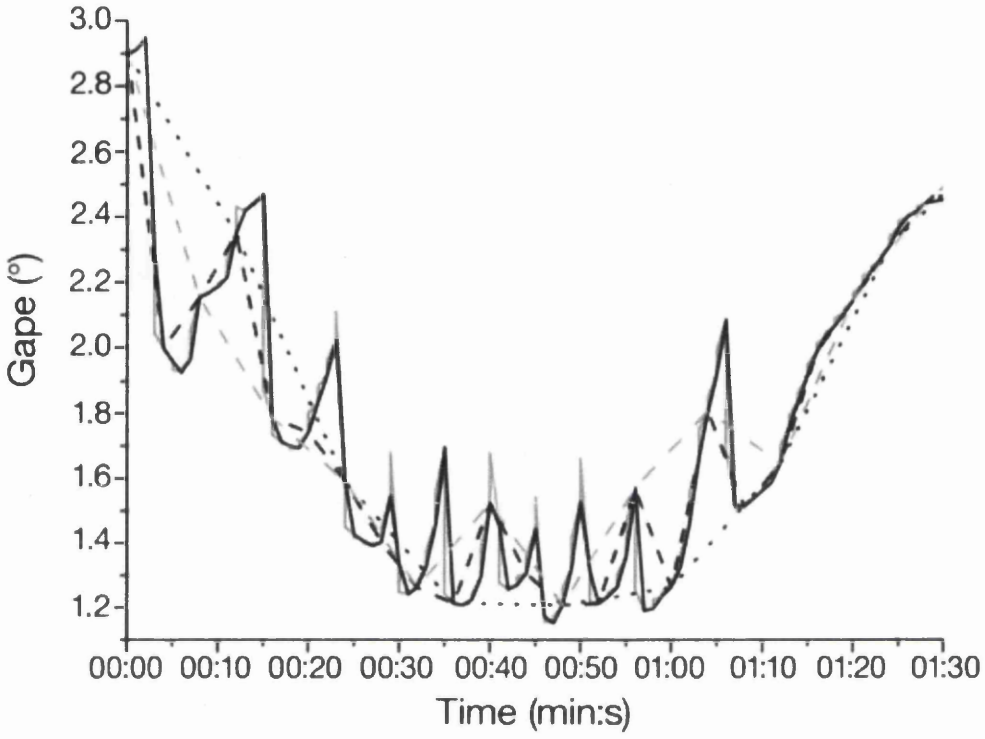


Fig.3.6. *Cerastoderma edule.* Example of the effect of sampling frequency on burrowing gape data from a 30 mm long cockle in an aquarium at Swansea University, UK. Sampling occurred once every 0.5 s (- - -), 1 s (—), 4 s (----), 8 s (– – –) and 12 s (• • •).

**Fig 3.7.**

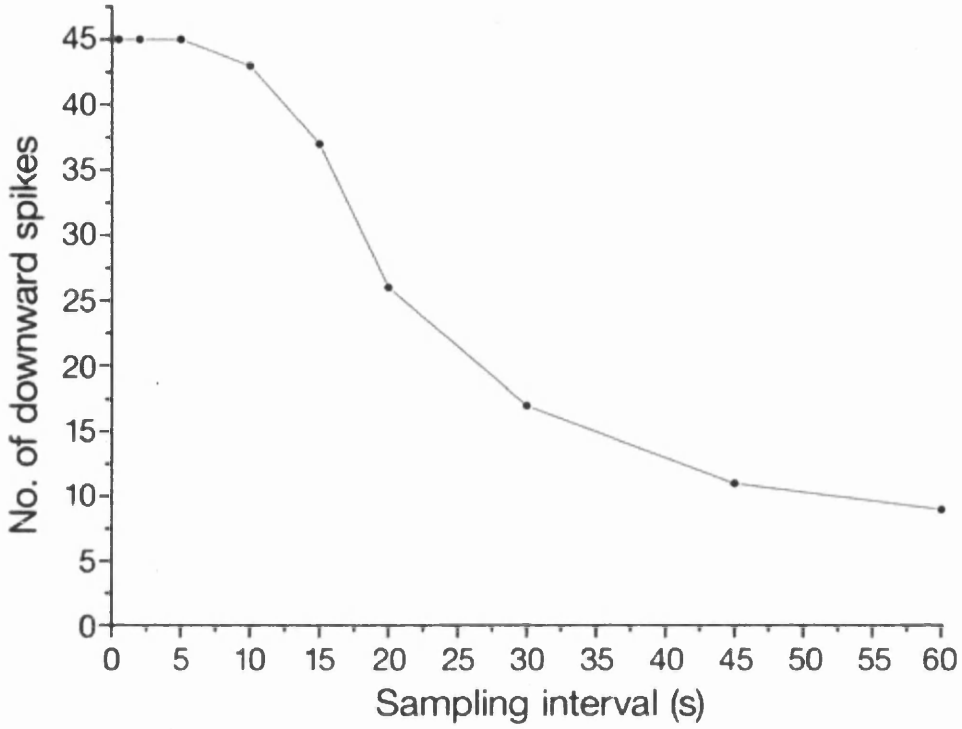


Fig. 3.7. *Margaritifera margaritifera.* Example of the effect of sampling interval on the number of detected downward spikes (i.e. valve adduction and subsequent abduction events) during 1 h of burrowing gape behaviour of a 105 mm long, freshwater pearl mussel in an aquarium.

**Fig 3.8.**

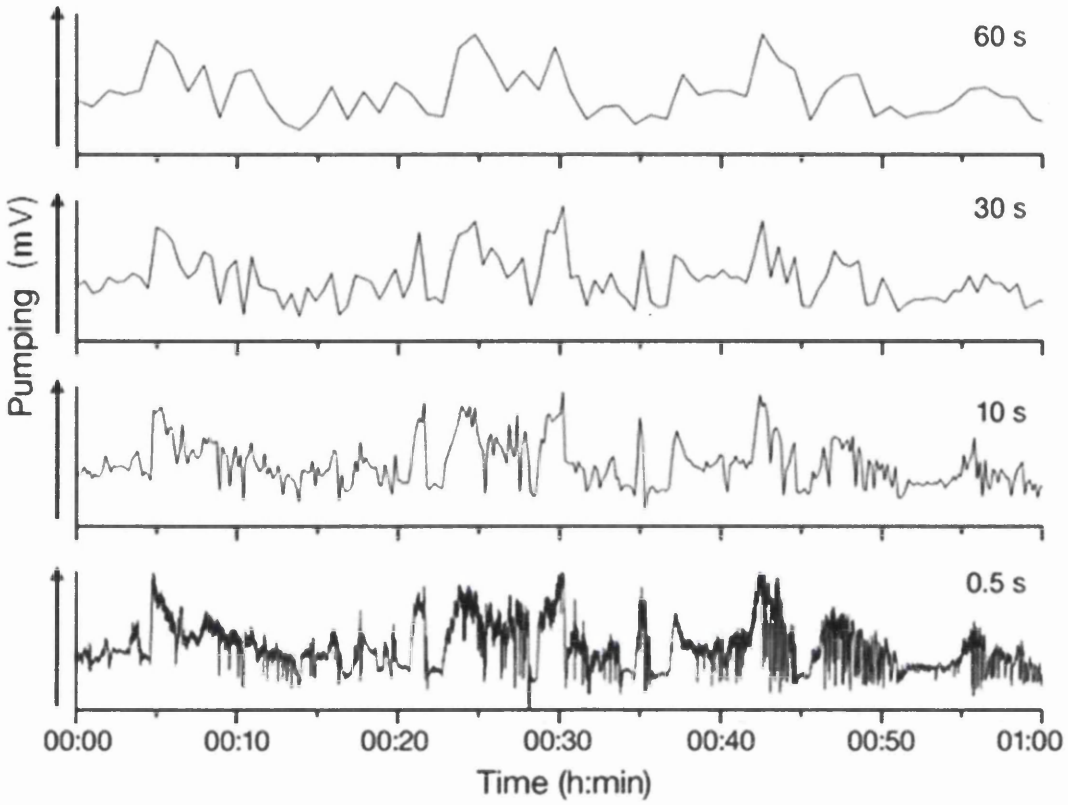


Fig. 3.8. *Mytilus edulis*. Example of the effect of sampling frequency on the exhalant pumping data from a 70 mm long mussel in an aquarium at Swansea University, UK. Sampling frequencies: 0.5, 10, 30 and 60 s.

**Fig 3.9.**

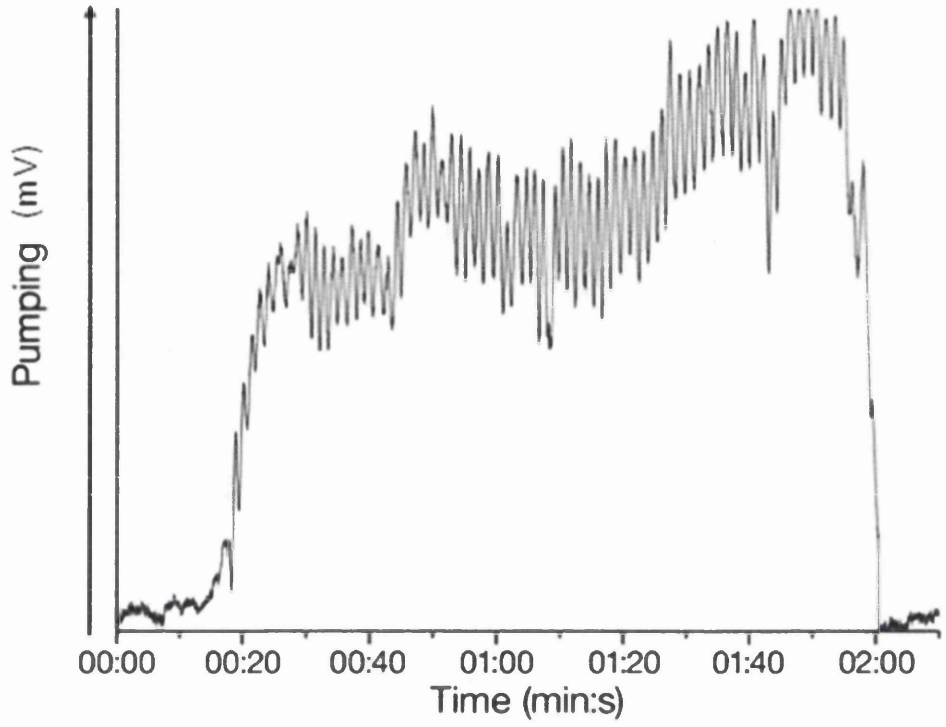


Fig. 3.9. *Mytilus edulis*. Example of a 75.5 mm long mussel eliminating faeces from the exhalant siphon in a seawater aquarium at Swansea University, UK. Elevated pumping activity (as observed by increased milivolt trace) is followed by a sharp decrease as the mussel closes its valves. Pumping was recorded at 30 Hz with a metachronal wave evident in pumping data.

**Fig 3.10.**

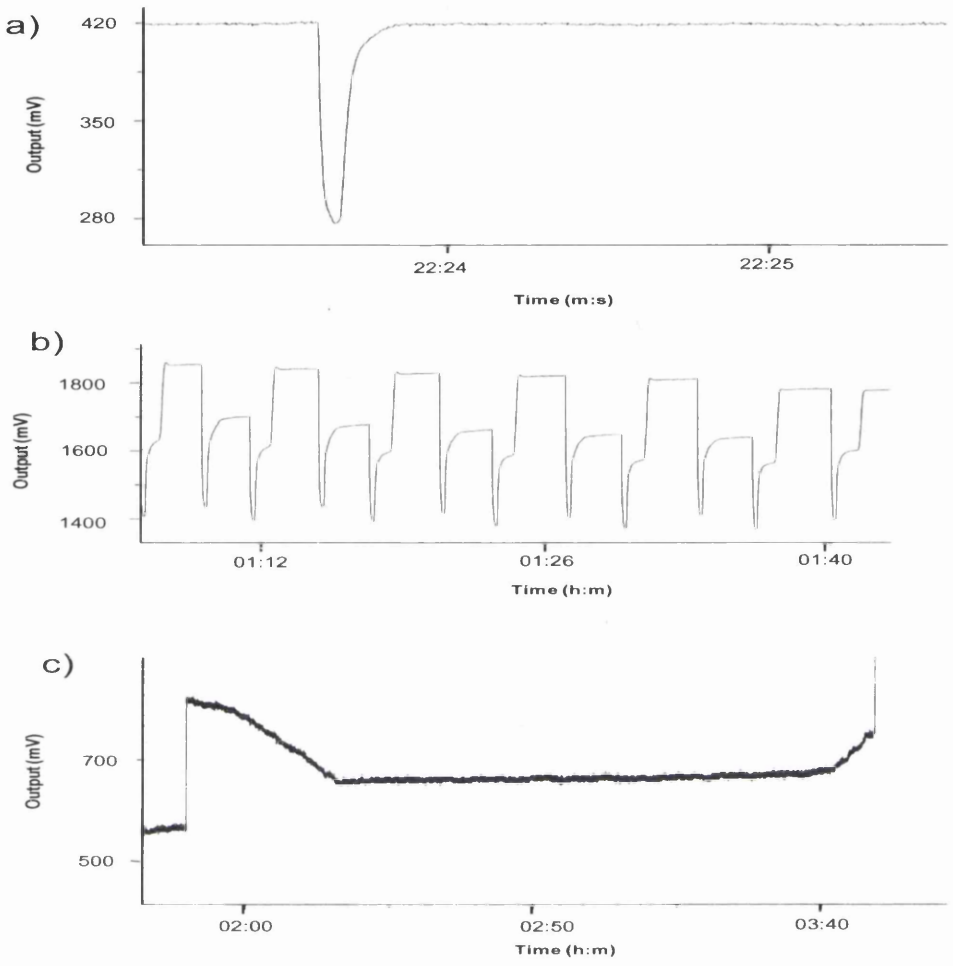


Fig. 3.10. Example of extended valve opening in a 100 mm long *Margaritifera margaritifera* recorded at a sampling frequency of 5 Hz. Figure 3.10 a) short (1 – 5 sec.); Figure 3.10 b) medium (minutes) and Figure 3.10 c) longer (hours) time periods. The short and medium duration events are composed of repeated open/close events, whilst the longer events are composed of period of extended opening.

# Chapter IV.

## Ghosts of hosts past – host specificity in the endangered freshwater pearl mussel *Margaritifera margaritifera*

Thomas, G.R. & Garcia de Leaniz, C. (under review) Ghosts of hosts past – host specificity in the endangered freshwater pearl mussel *Margaritifera margaritifera*. *Freshwater Biology*

# Chapter IV.

# Ghosts of hosts past – host specificity in the endangered freshwater pearl mussel, *Margaritifera margaritifera*


## ABSTRACT

Most studies of host-parasite systems deal with short-lived parasites that tend to evolve faster than their hosts. In contrast, very little is known about long-lived parasites that might be outpaced by their hosts. An experimental exposure approach was used to examine host specificity in the freshwater pearl mussel (*Margaritifera margaritifera*), an endangered bivalve that can live for over 100 yr. and which undergoes an obligate parasitic stage (glochidia) in the gills of suitable salmonid hosts. Glochidia prevalence differed significantly among salmonid hosts 15 days after encystment, being much higher for resident brown trout (*Salmo trutta*; 100%) and partially migratory arctic charr (*Salvelinus alpinus;* 100%) than for migratory Atlantic salmon (*Salmo salar;* 12.5%). Mean glochidia loads also differed significantly among salmonid hosts when statistically controlling for differences in body size, and were highest for resident brown trout ($m$ = 100.70, SE = 11.74), intermediate for partially migratory arctic charr ($m$ = 55.87, SE = 11.74) and lowest for migratory Atlantic salmon ($m$ = 0.208, SE = 0.120). No evidence of spleen inflammation was detected in any species, but glochidia cysts were significantly thicker, and encysted gill lamellae were more swollen relative to controls, in brown trout than in arctic charr. Results indicate that arctic charr remains a viable host for *M. margaritifera*, despite the fact that charr no longer cohabits with freshwater mussels in most British rivers since the last ice age. They also suggest that there may be important differences in glochidia susceptibility among salmonid hosts, being highest for resident brown trout and lowest for migratory Atlantic salmon, as predicted by models of host-parasite co-evolution. Variation in host response and susceptibility to parasitic glochidia should be taken into account when designing captive breeding and reintroduction programmes for the endangered freshwater pearl mussel.

**Keywords**: *Margaritifera margaritifera*; glochidia; host specificity; arctic charr; brown trout; Atlantic salmon

## INTRODUCTION

Co-evolution is a major force generating biodiversity (Thompson 1999) and host–parasite interactions constitute one of the best examples of co-evolution in spatially and temporally heterogeneous environments (Thompson 1994). In the evolutionary arms race, parasites can outpace their hosts by having larger population sizes, higher mutation rates, and shorter generation times, as these conditions typically result in greater evolutionary potential (Gandon & Michalakis 2002). Faster evolutionary rates by the parasite may lead to local parasite adaptations (LPA), but other factors such as gene flow, host range and metapopulation dynamics may also dictate the precise nature of host-parasite adaptations (Gandon & Michalakis 2002). Thus, high parasite dispersal should benefit the parasite, whereas high host dispersal should benefit the host (Gandon et al. 1996). In general, narrow host range, short parasite generation time and larger migration rate (relative to the host) are typically conductive of locally adapted parasites (Morgan et al. 2005), while greater host dispersal (relative to the parasite), and metapopulation dynamics should result in local host adaptations (LHA) due to evolutionary time lags (Lajeunesse & Forbes 2002). Most studies of host-parasite systems have focussed on short lived parasites leading to LPA, rather than on long-lived parasites that might lead to LHA, a situation which is not well understood (Gandon & Michalakis 2002; Lajeunesse & Forbes 2002).

An example of a extremely long-lived, specialist parasite is the freshwater pearl mussel (*Margaritifera margaritifera;* FWPM), an endangered unionid bivalve that can live for over 100 yr and which has an obligate parasitic stage attached to the gills of only two confirmed salmonid hosts across its range, the brown trout (*Salmo trutta*) and the Atlantic salmon (*S. salar;* Young & Williams 1984; Bauer 1987, 2001; Hastie & Young 2001; Hastie et al 2003). This host-parasite relation between salmonid hosts and the freshwater pearl mussel constitutes a particularly good system to examine local adaptations at the more controversial end of the host-parasite continuum because (a) the salmonid hosts' shorter generation time and migratory behaviour will tend to favour the

development of LHA, while (b) the parasite's (mussel) narrow host range will tend to favour the development of LPA. It is also a good system to understand adaptive responses to environmental uncertainty and climate change (Hastie et al 2003) since the host can move but the parasite cannot.

From a conservation perspective, parasites and mutualists are considered at a high risk of extinction due to their dependence on other species (Dunn et al 2009), while specialist organisms may be particularly at risk by a constrained response to rapid environmental change (Colles et al 2009). The conservation of specialist parasites with narrow host ranges, hence, is particularly challenging and would benefit from an evolutionary perspective. The historical distribution of the FWPM closely matches that of its salmonid hosts, and the species has suffered a marked decline, mirroring - and in some cases exceeding – salmonid host declines (Wells & Chatfield 1992; Hastie & Cosgrove 2001). With only two confirmed hosts, *M. margaritifera* has a particularly narrow host range compared to other unionid mussels (Bauer 2001; Wachtler et al 2001; Geist et al 2006). Recent declines in both FWPM and the Atlantic salmon (two of the most endangered aquatic organisms in Europe; Young et al. 2000; Hastie & Young 2003) stress the need for knowledge on the precise nature of the interaction between the FWPM and its hosts. While it has been suggested that brook trout (*Salvelinus fontinalis*) in eastern North America (Cunjak & McGladdery 1991), and Arctic charr (*Salvelinus alpinus*) in northern Europe (Bauer 1987), may also act as suitable hosts, this point has never been confirmed (Hastie & Young 2001).

The response of salmonid hosts to *M. margaritifera* infection is poorly known, despite the fact that glochidia encystment is necessary for the development of efficient conservation programs (based mostly on the artificial infection of salmonid hosts in captivity; Thomas et al 2010). Indeed, it has been suggested that the conservation of declining *M. margaritifera* populations must necessarily consider the interactions between mussels and their salmonid hosts (Geist et al 2006; Geist 2010). Mortalities of juvenile salmonids have been reported following artificial glochidia infection, and hatchery losses have sometimes been attributed to glochiodosis (Meyers & Millemann 1977; Treasurer et al. 2006), but in general, glochidia are thought to cause only minor damage upon salmonid hosts (Treasurer & Turnbull 2000; Treasurer et al. 2006). Yet,

fish hosts often display acquired humoral immunity following repeated exposure (Dodd et al 2006; Rogers-Lowery et al 2007), suggesting that glochidia of freshwater mussels represent some form of burden to the fish.

An experimental exposure of host fish to glochidia was conducted, followed by histological studies, to discriminate between local parasite adaptations (LPA) vs. local host adaptations (LHA). The objective of this study was to specifically test whether parasite infectivity (glochidia prevalence and loads) and host response (health condition) differed between migratory (Atlantic salmon) and resident (brown trout) salmonid hosts, as predicted by theories of host-parasite co-evolution. A secondary objective was to test whether Arctic charr, a salmonid that used to live sympatrically with freshwater mussels in rivers but which has since retreated to lakes in Britain after the last ice age, would still be a suitable host. The null hypothesis was that the longer generation time of the parasite and its lower dispersal capacity would result in LHA, (i.e. FWPM should perform better on resident than on migratory salmonid hosts). Under the LHA hypothesis, it can be expected that parasite fitness (as inferred from glochidia encystment rates) to be lowest among the migratory Atlantic salmon - with the highest dispersal rates - intermediate among arctic charr, and highest among the most sedentary brown trout.

## METHODS AND MATERIALS

### Experimental fish

Glochidia encystment was conducted at the Environment Agency Wales Cynrig Fish Culture Unit, near Brecon (Wales) between October and November 2008, as part of the EAW captive breeding programme for *M. margaritifera*. Juvenile 0+ Atlantic salmon (R. Taff stock; fork length 55-119 mm) and brown trout fry (R. Usk stock; fork length 54-130 mm) were derived from broodstock maintained at the EAW hatchery, whereas 0+ Arctic charr (fork length 104-161 mm) were derived from wild broodstock held at FRS Freshwater Laboratory, Perthshire, Scotland. Thirty three fish from each species ($n$ = 99) were transferred to a 1 x 0.5 x 0.5 m tank containing 50 adult mussels and kept for three days from 24$^{th}$ to 26$^{th}$ October 2008. We used mussels from a different river to the salmonid hosts (R. Wye) to avoid confounding effects due to potential host-parasite co-evolution at the river level, as we were interested in testing for host specificity at the species level, not the population level.

Whilst in the mussel holding tank, the fish were fed to satiation with a commercial pellet feed (Skretting). Following the 3-day cohabitation period, fish were transferred to a 2 m diameter tank (without mussels), where they were maintained for an additional 15 days and subsequently killed by an overdose of 2-phenoxyethanol. Daily mean water temperature was 8.0 °C (range 5.5–10.5 °C) and the estimated cumulative temperature units (TUs) was 176. Previous studies have shown that glochidia attachment is complete within 24-48 hr (Meyers & Millemann 1977; Araujo et al 2001); as such it can be confidently assumed that any glochidia remaining after 15 days must have been fully encysted.

### Glochidia counts

Gills were dissected and examined under a dissecting microscope (Leica) at x4 magnification, all glochidia were counted, and the first left gill arch placed in an excess of freshwater Bouin's fixative for subsequent histology (Humason 1979). Glochidia numbers were counted on two occasions separated several weeks apart to provide data on count repeatability from the same individuals. No false negatives were detected and

repeatability of glochidia counts was very high (intraclass-correlation coefficient = 0.999, Cronbach's Alpha = 1.000).

**Spleen area and gill histology**

Spleens from salmonid hosts with varying glochidial loads were dissected and photographed with a Canon EOS D40 fitted with a SIGMA EM-140 DG ringflash and a macro lens (TAMRON SP DI 90 mm 1:2.8, 1:1 magnification), mounted on a copy stand at a fixed 40 cm height from the object. Spleen areas were subsequently digitized from high resolution TIFF images using Image-J (Abramoff et al. 2004) in order to test for glochidia-induced splenomegaly (enlargement of the spleen). As with glochidia loads, repeatability in measurements of spleen area was very high (intraclass-correlation coefficient = 0.999, Cronbach's Alpha = 1.000).

Histologically-fixed gill arches were dehydrated in a series of graded ethanol baths (70, 80, 90 and 100%), and cleared with Histoclear before mounting in paraffin wax. Serial sections (6 μm) were made using a 52164 Kent Cambridge rotary microtome and at least 10 slides per individual were stained using the haemotoxylin-eosin method (Lillie 1965). Gill sections were then photographed using an Olympus C500 digital camera mounted on an Olympus BX41 microscope at x40 magnification. The width and length of one control (without encysted glochidia) and one encysted secondary lamellae, as well as the thickness of the cyst wall at 0°, 180° and 270° axes were measured for each individual host from high resolution digital photographs using Image-J (Fig. 4.1). The number of mucous cells in a standard 200 $\mu m^2$ rectangle centred on the cyst was also counted.

**Statistical analysis**

Differences in glochidia prevalence among the three salmonid hosts were tested by the log-likelihood ratio test. A backward stepwise multiple regression was employed to examine the relationship between body size (fork length) and species identity on glochidia loads, as well as between glochidia loads and species identity on splenic index. ANCOVA was used to test for variation in mean glochidia loads (*m*) among hosts while statistically controlling for variation in body size. Glochidia-induced changes in

gill morphology were tested in two different ways, by comparing the size (length and width) and density of mucous cells of encysted and control (unencysted) lamellae from the same individuals, and by directly measuring cyst wall thickness (as a measure of inflammation) along the 0°, 180° and 270° cyst axes. In both cases, MANCOVA was employed to compare differences between hosts while statistically controlling for variation in host body size. SPSS 16.0 and SYSTAT v. 10 were used for all statistical tests, and logarithmic transformation was applied to improve normality and homogeneity of variances, as required.

## RESULTS

### Glochidia prevalence

Glochidia were found encysted on the gills of all three salmonid species, albeit at very different frequencies ($G$ = 70.196, df = 2, $P < 0.001$). Thus, glochidia prevalence after 15 days post exposure was much higher for brown trout (27/27 or 100%) and arctic charr (23/23 or 100%) than for Atlantic salmon (3/21 or 12.5%).

### Effect of host body size on glochidia loads

Stepwise multiple regression indicated that glochidia loads (log transformed) depended on the interaction between body size (log transformed) and host identity ($F_{2,70}$ = 142.070, $P < 0.001$). Hence, for juvenile brown trout and arctic charr, larger fish tended to harbour more encysted glochidia in their gills than smaller fish of the same age, but such an effect was not evident for juvenile Atlantic salmon (Figure 4.2).

### Glochidia loads

Mean glochidia loads ($m$) differed significantly between hosts when statistically controlling for differences in body size (Figure 4.3; ANCOVA, $F_{2,70}$ = 13. 13.584, $P <$ 0.001), and were highest for brown trout ($m$ = 100.70, SE = 18.62), intermediate for arctic charr ($m$ = 55.87, SE = 11.74) and lowest for Atlantic salmon ($m$ = 0.208, SE = 0.120). Only brown trout and arctic charr were subsequently sampled for gill histology, as the low prevalence of glochidia on Atlantic salmon prevented further analysis for this species.

### Gill histology

Brown trout and arctic charr differed significantly in the extent of glochidia-induced changes in gill histology (MANCOVA Wilk's Lambda = 0.671, $F_{3,23}$= 3.760, $P$ = 0.025). Encysted lamellae in both species were significantly more enlarged and contained fewer mucous cells compared to control lamellae, but the changes were more pronounced in brown trout than in arctic charr (Table 4.1). Post-hoc univariate tests revealed that the main difference between salmonid hosts rested in the much more pronounced increase in the width of encysted lamellae amongst brown trout ($F_{1,25}$ =

10.878, $P = 0.003$), rather than in differences in lamellae length ($F_{1,25} = 0.396$, $P = 0.535$) or in density of mucous cells ($F_{1,25} = 0.043$, $P = 0.837$), which changed similarly in response to glochidia encystment in both host species. Direct comparisons of encysted glochidia confirmed these differences in the extent of lamellae swelling between species. Thus, the host tissue response around glochidia was significantly thicker in brown trout than in arctic charr (Figure 4.1), when body size was statistically controlled for (MANCOVA Wilk's-Lambda = 0.166, $F_{3,26} = 43.694$, $P < 0.001$; post-hoc univariate ANOVAs at 0° axis $F_{1,28} = 32.671$, $P < 0.001$; 180° axis $F_{1,28} = 28.777$, $P < 0.001$; 270° axis $F_{1,28} = 73.942$, $P < 0.001$).

**Splenomegaly**

Relative spleen weight varied significantly among salmonid hosts ($F_{2,67} = 153.722$, $P < 0.001$) and arctic charr had spleens that were much heavier for their size than those of juvenile salmon or brown trout (Bonferroni-adjusted pairwise comparisons $P < 0.001$). However, relative spleen size was unrelated to glochidia loads ($F_{1,67} = 0.000$, $P = 0.998$) or to the interaction between host species and glochidia loads ($F_{2,67} = 0.052$, $P = 0.949$). The same results were obtained if juvenile Atlantic salmon (most of which had no glochidia) were excluded. Thus, there was no indication that glochidia encystment resulted in enlarged spleens 15 days post-exposure in any of the three host species (Figure 4.4).

## DISCUSSION

The long life-span and complex life histories of freshwater pearl mussels make their conservation particularly challenging, and better knowledge on the extent of host specificity has been highlighted as a research priority for the development of conservation and artificial propagation programmes (Cosgrove & Hastie 2001; Strayer et al. 2004; Geist & Kuehn 2005). To our knowledge, our study represents the first direct exposure study to address host specificity in *M. margaritifera,* and the first report to show that the glochidia of the freshwater pearl mussel can successfully attach to arctic charr (*Salvelinus alpinus*) and survive for 15 days. Although our sample sizes are admittedly small, and the monitoring period relatively brief, three lines of evidence would suggest that arctic charr is indeed a viable host for *M. margaritifera*: (1) among unsuitable fish hosts, glochidia of freshwater mussels are sloughed away typically within 48 – 72 hours (Dodd et al 2005; Rogers-Lowery & Dimmock 2006; Rogers-Lowery et al 2007), whereas 100% encystment rate was found in arctic charr in our study 15 days after exposure, the same as for brown trout, (2) histologically, glochidia attached to arctic charr were well developed and fully encysted, and (3) average glochidia load in the gills of arctic charr was half that of brown trout, but over 260 times higher than that observed in Atlantic salmon, a common host of the freshwater pearl mussel. As there are no extant populations of arctic charr in most British rivers (Klemetsen et al 2003), it is assumed that *M. margaritifera* would not have had contact with this host since the last ice age. On the other hand *S. alpinus* still coexists with *M. margaritifera* in a few Scottish rivers (Walker 2007), providing an opportunity for glochidia to encyst on riverine arctic charr further north.

Glochidia of freshwater mussels can attach to several fish species, but successful development and larval transformation is typically only possible on a few specific hosts (Fustish & Millemann, 1978; Karna & Millemann, 1978; Bauer & Vogel 1987). *M. margaritifera* appears to have a narrower host range than most unionids (Bauer 2001), a fact that has, perhaps, exacerbated its decline (Arajuo & Ramos 2001; Hastie & Cosgrove 2001; Hastie & Young 2003). However, only a handful of fish hosts have been experimentally tested. While there has been much research on host specificity of North American unionids (Strayer 2008), the hosts of *M. margaritifera* are believed to

be confined to the Salmonidae (Young & Williams 1984; Bauer 1987; Cunjack & McGladdery 1991; Hastie & Young 2003; Geist et al 2006). Yet, non–salmonids such as *Acipenser baeri, A. sturio* and *Salaria fluvitalis* represent suitable hosts for the closely related *M. auricularia* (Araujo et al 2001; Lopez et al 2007), *Noturus phaeus* is a suitable host for *M. hembeli* (Johnson & Brown 1998), while the host of *M. marocana* is yet to be ascertained (Araujo et al 2009). Clearly, closely related margaritiferids are able to utilise different fish genera as hosts, suggesting that additional fish hosts for *M. margaritifera* may well yet to be discovered.

Our results indicate that, with the exception of salmon which were only rarely infected in our study, larger salmonid hosts tended to harbour more glochidia than smaller hosts. This suggests that glochidia attachment is, at least initially, a function of gill area. A positive association between body size and parasite loads has previously been noted for several fish species, due to larger fish having relatively larger surface area and higher feeding rates, factors that would tend to favour parasite exposure (Poulin 2000). As captive breeding programmes for freshwater mussels often aim for high encystment rates in order to maximise the number of mussels produced (Thomas et al 2010), our study suggests that it may be beneficial to select the largest fish as hosts. However, large fish may also shed greater numbers of glochidia, and a potential trade-off may exist between encystment rates and transformation success, which would merit further study.

All brown trout and arctic charr in our study were encysted with glochidia, compared to only 12.5% of Atlantic salmon, despite the fact that fish were exposed to adult mussels simultaneously, in a common tank, and for the same period of time. As the potentially confounding effect of body size was accounted for, the differences in encystment rate and glochidia loads observed among salmonid hosts are probably real, and likely represent differences in anti-glochidial response. Indeed, fish hosts are known to differ widely in anti-glochidial antibodies (Meyers et al 1980; Bauer & Vogel 1987; O'Connell & Neves 1999), and such differences are manifested in varying encystment rates. For example, *Lepomis macrochirus* which had developed an acquired immunity to the glochidia of *Utterbackia imbecillis* produced thinner and incomplete cysts (Rogers & Dimock 2003), whilst previously exposed *Micropterus salmoides* shed glochidia

faster than naive fish when exposed to the glochidia of *Lampsilis reeveiana* (Dodd et al 2005). These, and other studies (Reuling 1919; Arey 1924; Bauer & Vogel 1987; Dodd et al 2006; Rogers-Lowery et al 2007) suggest that glochidia encystment is mediated by an antigen-antibody host response.

Parasitic infection often results in enlarged spleens in many animals (Moller 1998; Moller & Erritzoe 1998) due to immunologically-mediated responses (Brown & Brown 2002). In fish, the spleen is the major organ of the immune system and the location of soluble antigen recognition (Rowley et al 1999), so one might also expect an enlargement of the spleen of salmonids following glochidial encystment. However, in our study splenomegaly was not observed in any of the three salmonid hosts at 15 days post exposure, perhaps suggesting that a full humoral immune response had not yet been mounted. However, as Rogers-Lowery et al (2007) have noted, fish hosts can mount both humoral and mucosal antibody responses to glochidia encystment, and the timing of such responses can vary over the course of infection. On the other hand, comparative gill histology showed clear signs of gill inflammation, as well as a significant depletion of mucous cells amongst encysted secondary lamellae, such changes being more pronounced in brown trout than in arctic charr. Host cysts surrounding glochidia were also significantly thicker in brown trout than in arctic charr, a factor that may reduce sloughing (Fustish & Millemann 1978; Araujo et al 2001), and which may explain the higher glochidia loads observed in trout than in charr.

The three salmonid hosts used in our study tend to occupy different positions along a dispersal continuum, brown trout being typically resident, arctic charr being intermediate, and Atlantic salmon being clearly the most migratory of the three species (Klemetsen et al 2003). Taken together, our results suggest that the most suitable host for *M. margaritifera* is the resident brown trout, whilst the migratory Atlantic salmon appears to be the most resistant to glochidiosis. Arctic charr, a species which migrates between rivers and lakes and which will therefore disperse more than most trout but less than most salmon, also appears to be a suitable host, although it tended to form thinner cysts and harboured less glochidia than brown trout. The dispersal capability of host fish, therefore, appears to play an important role in determining the success of *M. margaritifera* encystment, as predicted by models of host-parasite co-evolution (Gandon

et al. 1996; Gandon & Michalakis 2002; Morgan et al. 2005; Lajeunesse & Forbes 2002). Uniquely, members of the Unionoidea also rank amongst some of the longest-lived aquatic invertebrates in the world (Anthony et al 2001). For example, *M. margaritifera* attains sexual maturity after 12-20 years (Young & Williams 1984), can live in excess of 100 years (Bauer 1992), and will therefore outlive its fish hosts. More generally, our study suggests that in the salmonid-mussel host-parasite system the longer generation time of the parasite and its lower dispersal capacity has probably resulted in local adaptation by the host (LHA), rather than in the more common local adaptation by the parasite (LPA). This would also explain why *M. margaritifera* appears to perform better on resident than on migratory salmonids. Immuno-genetic studies, like those carried out with other salmonid parasites (e.g. Consuegra & Garcia de Leaniz 2008), appear warranted and should provide a unique insight into the adaptive responses of different fish hosts to glochidiosis, as well as into the evolutionary arms–race that has shaped such unusual host-parasite system.

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Table 4.1. ANOVA results for comparative glochidia-induced changes in the secondary lamellae of juvenile arctic charr (*Salvelinus alpinus;* n = 16) and brown trout (*Salmo trutta*; n = 15) 15 days post-exposure (176 cumulative temperature units). * denotes significant differences between hosts.

| Variable | *Salvelinus alpinus* | *Salmo trutta* | ANOVA | | |
|---|---|---|---|---|---|
| | Mean (SD) | Mean (SD) | df | *F* value | *p* |
| Lamellae width (μm) | | | | | |
| Control | 195.6 (41.5) | 141.4 (50.7) | 25 | 10.878 | 0.003 * |
| Encysted | 259.5 (46.2) | 251.6 (46.8) | | | |
| Lamellae length (μm) | | | | | |
| Control | 354.8 (98.0) | 245.20 (76.2) | 25 | 0.396 | 0.535 |
| Encysted | 396.0 (46.6) | 298.9 (78.4) | | | |
| No. mucous cells/ 200 $\mu m^2$ | | | | | |
| Control | 5.6 (2.3) | 3.4 (3.0) | 25 | 0.043 | 0.837 |
| Encysted | 2.7 (2.7) | 0.4 (0.5) | | | |
| Cyst wall thickness (μm) | | | | | |
| 0° axis | 17.9 (9.6) | 67.5 (31.2) | 28 | 32.671 | <0.001* |
| 180° axis | 11.5 (4.7) | 39.3 (18.9) | 28 | 28.777 | <0.001* |
| 270° axis | 18.5 (10.1) | 83.2 (27.6) | 28 | 73.942 | <0.001* |

**Fig. 4.1**

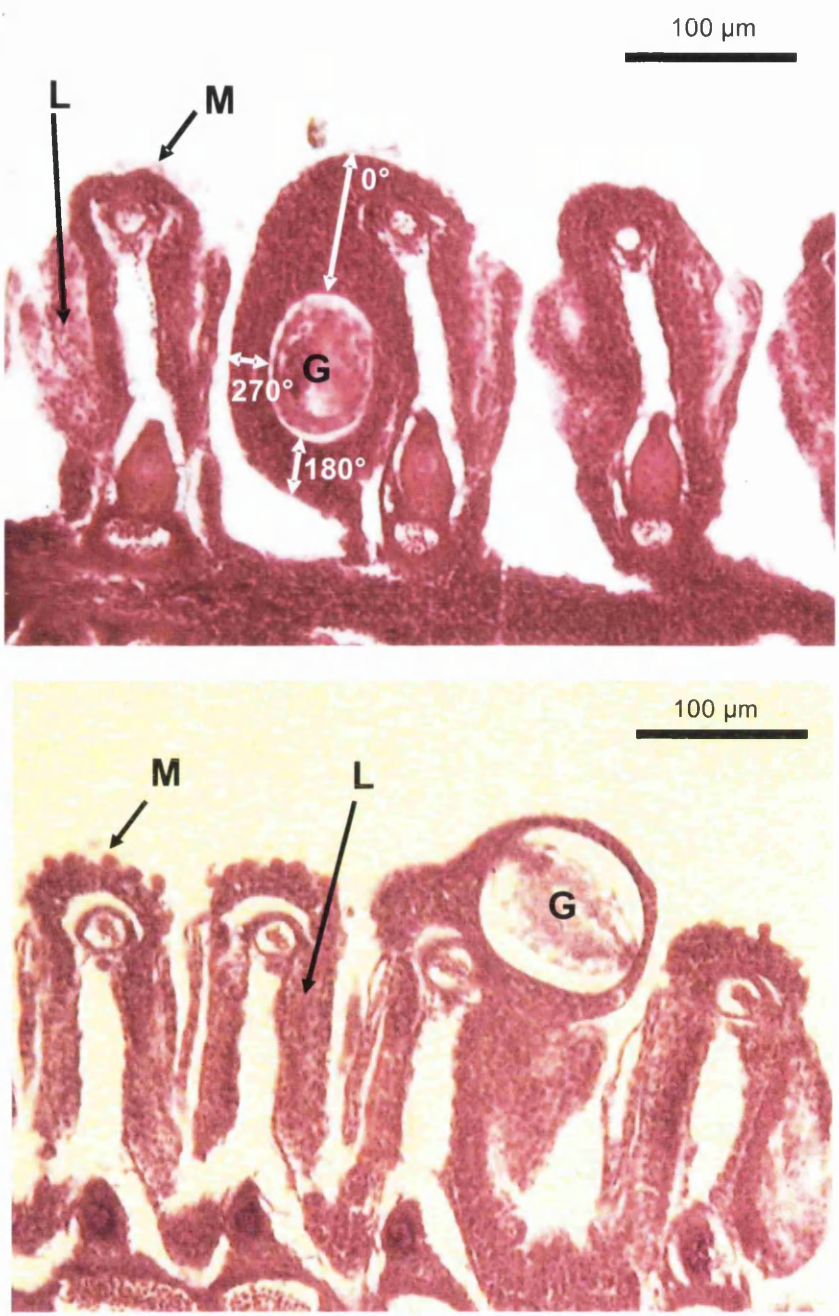


Figure 4.1. Encysted *M. margaritifera* glochidia in the gills of (a) brown trout, *Salmo trutta* and (b) arctic charr, *Salvelinus alpinus* 15 days post-exposure (176 cumulative temperature units). Key – G glochidia, L secondary lamellae, M mucous cells. H & E stain, 10x magnification. Arrows denote the three axes used for measurement of cyst wall thickness.

**Fig. 4.2**

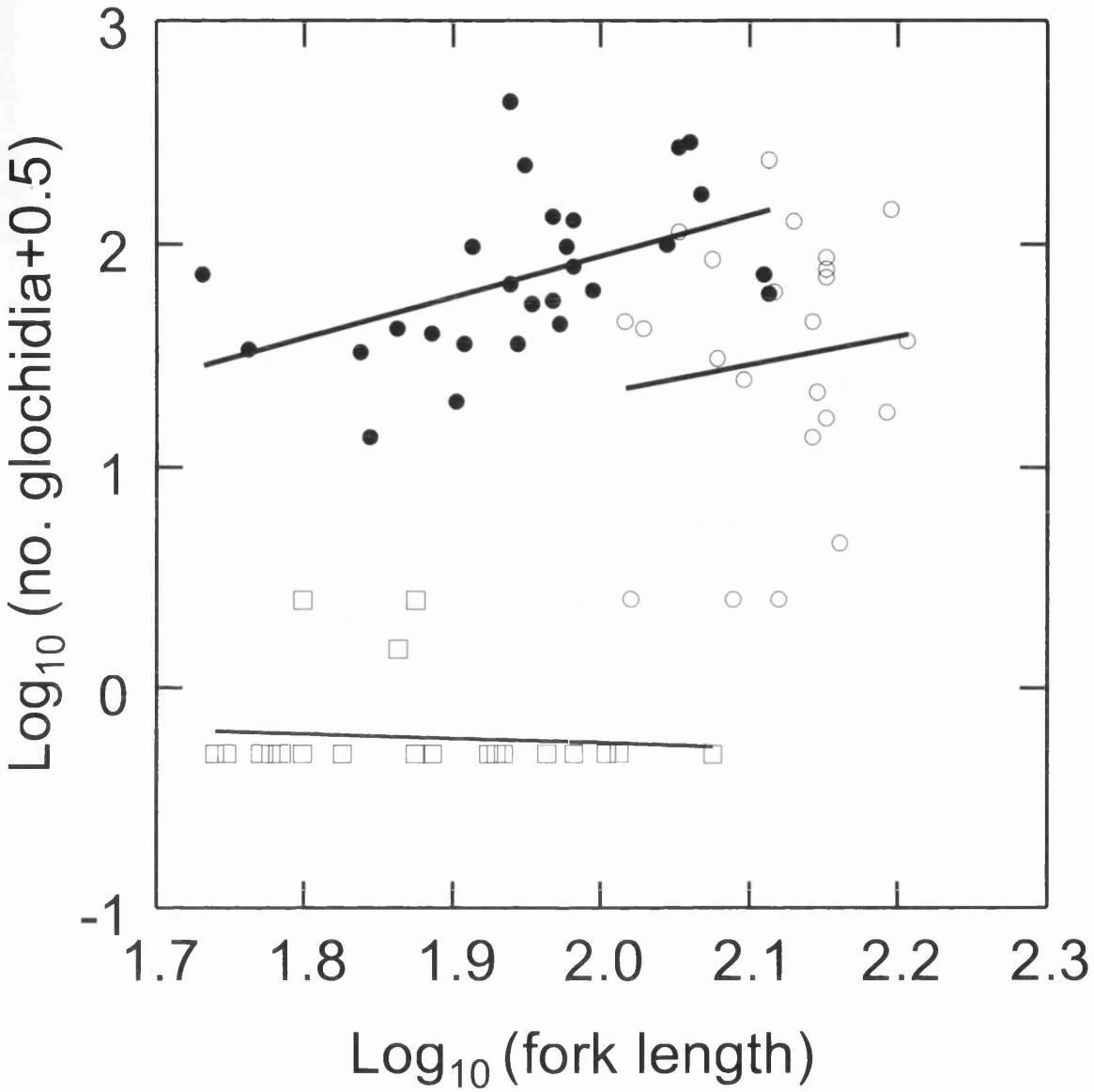


Figure 4.2. Relationship between host body size (fork length) and *Margaritifera margaritifera* glochidia loads in juvenile brown trout (●), Atlantic salmon (□), and Arctic charr (○) 15 days post exposure (176 cumulative temperature units).

**Fig. 4.3**

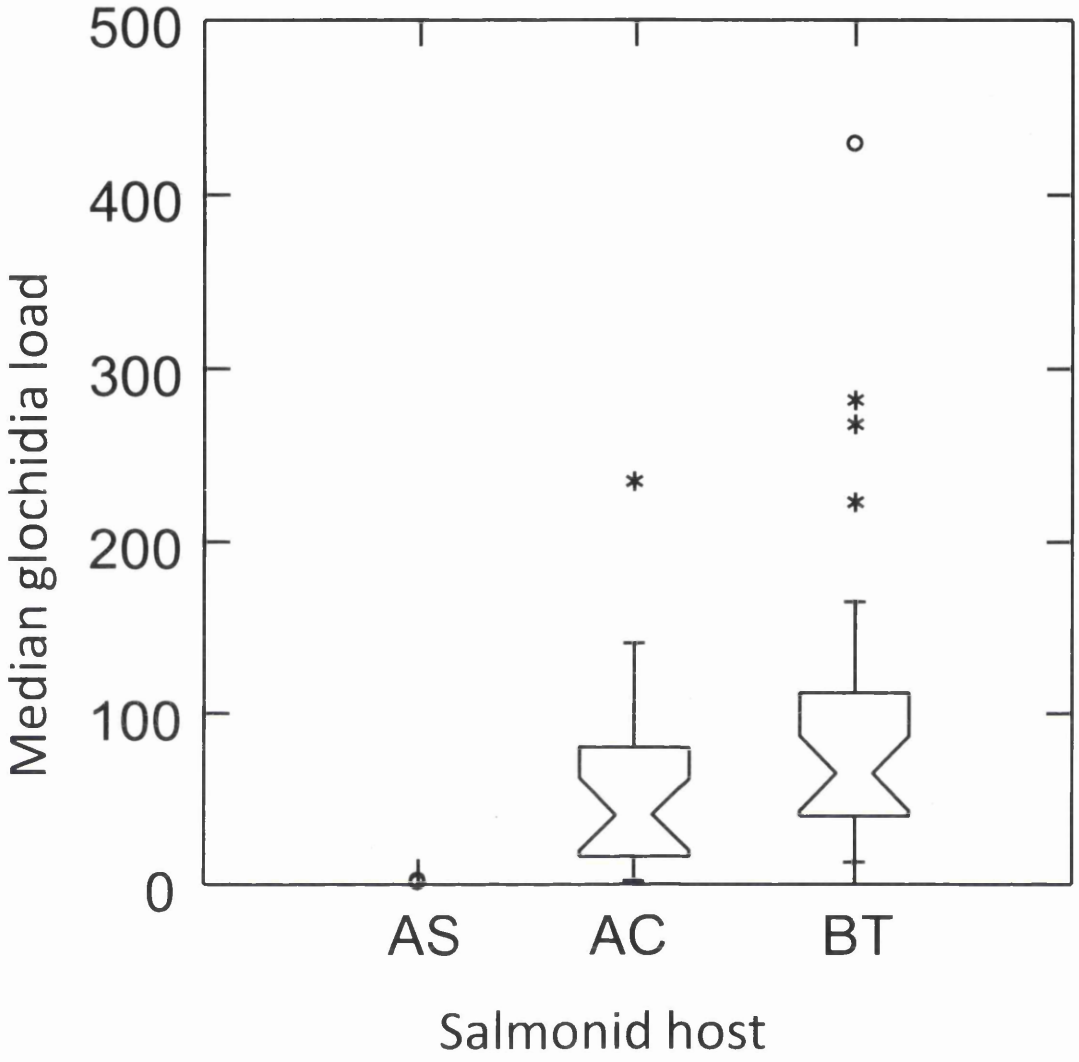


Figure 4.3. Variation in *Margaritifera margaritifera* glochidia loads among three salmonid hosts (AS, Atlantic salmon; AC, Arctic charr; BT, brown trout) 15 days post exposure (176 cumulative temperature units). Box plots show median values with notches extending to 95% CI around the median, first and third quartiles (boxes), 90% of values (whiskers) and extreme data points (asterisks and circles).

**Fig. 4.4**

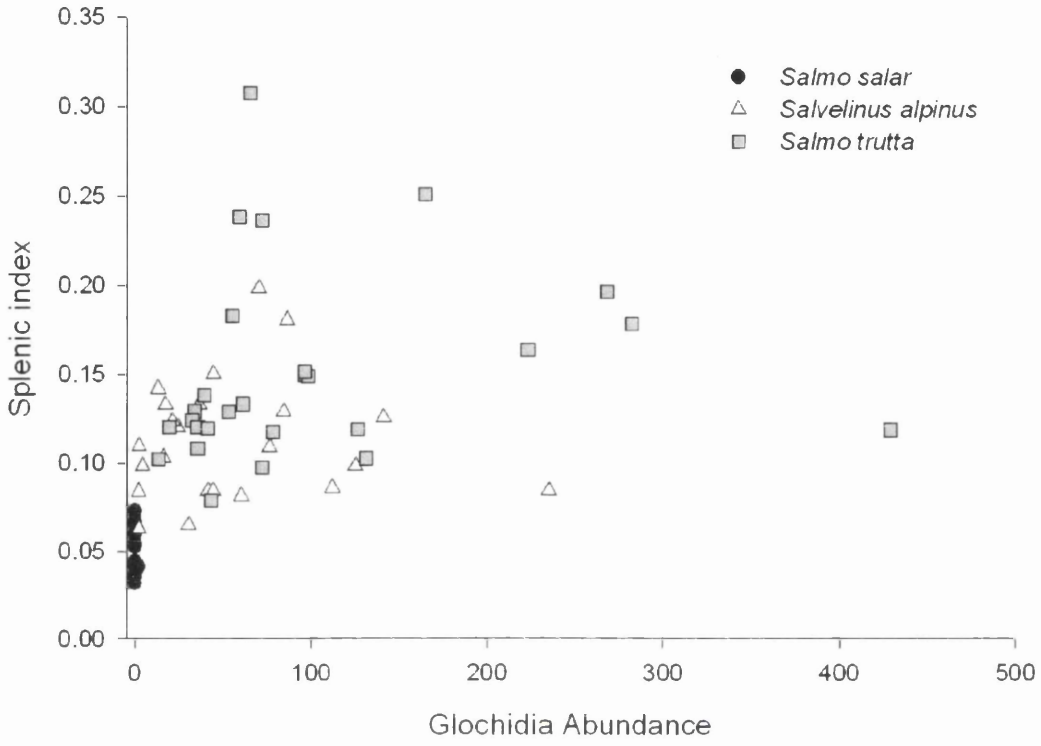


Figure 4.4. Relationship between glochidia load and splenic index (relative spleen weight) of three salmonids hosts 15 days post exposure (176 cumulative temperature units).

# Chapter V.

## Physiological effects of *Margaritifera margaritifera* on brown trout *Salmo trutta*

# Chapter V.

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## ABSTRACT

The physiological response of juvenile brown trout (*Salmo trutta*) to glochidia encystment of the freshwater pearl mussel, *Margaritifera margaritifera*, was examined at various times post-exposure. Glochidia abundance was positively correlated to host body size and was accompanied by significant spleen enlargement at 31 days post-exposure, but not before (15 days) of after (160 days). No significant differences in mean blood haematocrit or in ventilation frequency (measured as opercula beat rate) were detected between encysted and uninfected fish, once the effects of body size had been statistically accounted for. Opercular beat rate was significantly related to host body size, but not to glochidia prevalence or abundance. The cryptic colouration of the host, measured as the intensity and contrast of lateral parr markings, was also unrelated to glochidia prevalence or abundance. Our results suggest that the physiological impacts of glochidiosis on juvenile brown trout are probably slight, and that although an anti-glochidial immune response was probably mounted by the fish, this appears to be short-lived and to peak at one month post-exposure.



**Keywords:** brown trout, freshwater mussel, physiology, splenomegaly, crypsis, respiration.

## INTRODUCTION

Freshwater mussels (Bivalvia: Unionoidea) are often considered to be amongst the most endangered aquatic organisms (Lydeard et al 2004; Strayer et al 2004), and are the target of conservation programmes in several countries (Thomas et al 2010). Unionid mussels have an obligate parasitic stage attached to the gills or fins of freshwater fish, known as glochidia. Glochidia encyst on host tissues and remain attached to the host for varying periods of time, a condition known as glochidiosis (Meyers & Millemann 1977). During the course of encystment, fish are thought to mount an immune response (Meyers et al 1980; Bauer & Vogel 1987; O'Connell & Neves 1999), which results in the shedding of large numbers of glochidia (Hastie & Young 2003). However, very little is known about the effects of glochidia on fish hosts, although it is assumed that it must represent some form of burden to the fish (Treasurer & Turnbull 2000; Treasurer et al 2006) and that it is therefore advantageous for the host to remove as many glochidia as possible.

Several traits of the fish hosts can influence the prevalence and abundance of parasites they will have, of which body size has sometimes been found to be influential (Poulin 1995; 2000). Thus, some authors have found a negative correlation between glochidia abundance and fish body size (Bauer 1987b), whilst others found no such correlation (Cunjack & McGladdery 1991; Beasley 1996; Treasurer & Turnbull 2000; Treasurer et al 2006). However, host responses can also depend on previous glochidia exposure (which can lead to acquired immunity; Bauer & Vogel 1987; O'Connell & Neves 1999; Rogers-Lowery et al 2007), as well as on time post-exposure, as host responses to glochidia can vary over the course of encystment (Young & Williams 1984; Hastie & Young 2001). The initial report of "acquired immunity" against glochidia was made by Reuling (1919). Since, then several authors have suggested that a humoral immune response may be responsible for causing immunity against glochidial infections in fish (Fustish & Millemann 1978; Bauer 1987a; Bauer & Vogel 1987; Rogers-Lowery et al 2007). The presence of anti-glochidial antibodies have been noted in fish encysted with glochidia (Bauer & Vogel 1987; O'Connell & Neves 1999; Rogers-Lowery et al 2007). Thus, it is important to consider host responses at different

tines over the course of infection, and also to use hosts which have not had previous glochidia exposure.

As the spleen of fish is the location of soluble antigen recognition (Rowley et al 1999), spleen enlargement (splenomegaly) can sometimes be related to parasite load (Brown & Brown 2002). Depending on the type of parasite, parasites can also have an affect on blood parameters, of which the haematocrit or packed red cell volume is perhaps the easiest to measure (Woo 1969). Reduced haematocrit values have been reported in many parasitised organisms, including Rusa deer (*Cervus timorensis russa*) infected by the trypanosome *Trypanosoma evansi* (Reid et al 1999), blackeye thicklip infected by gnathid isopods (Jones & Grutter 2005), and rabbitfish *Siganus luridus* infected by the microcotylid *Allobivagina* spp. (Paperna et al 1984), amongst many others. However, a reduction in haematocrit is not always observed in parasitised hosts (Gibson 1990).

With respect to the glochidia of freshwater mussels, it is unclear if glochidiosis has an impact on haematocrit values. Glochidia of the margaritiferids form cysts in the fish secondary gill lamellae that pierce the host's blood vessels (Karna & Millemann 1978; Araujo & Ramos 1998; Araujo et al 2002), but whether glochidia depend on a blood supply from the host, or have an effect on host physiology, is unclear. Fisher and Dimock (2002) describe the digestion of enclosed host gill tissue by the encysted larvae of *Utterbackia imbecillis*, but others (Barnhart et al 2008) regard the relationship between glochidia and the fish host as being predominantly phoretic, with little or no feeding taking place during encystment. Thus, the effect of glochidiosis on haematocrit values may give insights into the burden glochidia may exert on their host.

Similarly, very little is known about the impacts of glochidiosis on the hosts' respiratory capabilities. The glochidia of *M. margaritifera* lack hooks and are exclusively gill parasites (Wächtler et al. 2000). Fusion of secondary lamellae, nodule formation, and a thickening or scarring of the gills have been noted following excystment of glochidia, and these may increase resistance to gas diffusion, and perhaps decrease respiratory performance (Meyers et al 1980). Yet, very little information is available on the effect of glochiodosis on host respiratory performance, and none that we know of involving the freshwater pearl mussel. Several fish, including juvenile

Atlantic salmon (Hawkins et al 2004) and rainbow darters (Gibson & Mathis 2006), increase their ventilation rate in the presence of predator cues, and this is believed to facilitate a escape response (Lydersen & Kovacs 1995; Hawkins et al 2004). Glochidia encystment could, therefore, have an effect on the hosts' ventilation rates, which could in turn affect its anti-predatory performance.

In common with other teleosts, salmonid hosts have evolved physiological adaptations to reduce the risk of predation (Leclercq et al 2010), including the evolution of cryptic colouration (Donnelly & Dill 1984; Endler 1986; Bond & Kamil 2002; Seppala et al 2005; Stevens & Merilaita 2009). Some parasites can disrupt host crypsis in order to make the host more conspicuous to predators, thereby facilitating the parasites' transmission to the next host (reviewed in Moore 2002). Glochidia, however, are not trophically-transmitted parasites. Indeed, their relationship with fish has variously been described as either phoresy (Barnhart et al 2008), or even as a form of symbiosis-protocooperation (Geist 2010). As such, therefore, we would not expect to detect major impacts of glochidia upon the salmonid hosts, if these were to decrease the mussel's chances of surviving before excystment from the host. With this in mind, we examined several aspects of glochidiosis on the physiology of brown trout exposed to the glochidia of *M. margaritifera*. Our expectations were that (1) any impacts of glochidia on the host haematological parameters and respiratory performance would be mild before excystment, and that (2) glochiodosis would not disrupt the crypsis colouration of the host.

## METHODS

### Sources of fish and estimation of days post-exposure

Studies were conducted at the Cynrig Fish Culture Unit of the Environment Agency Wales (Powys, Wales) and at the Freshwater Research Unit, Swansea University. Juvenile 0+ brown trout (*Salmo trutta*) used in this study (fork length 54-202 mm) were derived from R. Usk broodstock maintained at the EAW hatchery, as part of the Environment Agency (Wales) captive breeding program for *M. margaritifera.* Approximately 1,000 fish were transferred to a 1.5 diameter tank which was connected to a holding tank containing 50 adult mussels from the R. Wye population at least 2 months before glochidia spatting during the autumns of 2008 and 2009. The approximate dates of spatting (glochidial release) were estimated from information on the dates when glochidia were first found on fish. Thus, no glochidia were found on fish sampled on the 9$^{th}$ September 2008, but were present on fish sampled on the 6$^{th}$ of October 2008. The mid-point date (22$^{nd}$ September) was thus taken to be the date of glochidial release for 2008. Likewise, no glochidia were found on fish sampled on 21$^{st}$ September 2009, but were present on fish sampled on the 6$^{th}$ of October, giving the 28$^{th}$ September 2009 as the estimated date of glochidial release for 2009. Days post-exposure (d.p.e) were then calculated for these estimated dates of glochidial release (Table 5.1).

### Estimation of glochidia abundance

At each sampling period, a sample of 27-90 juvenile brown trout were humanely killed by an overdose of anaesthesia, weighed (wet weight, 0.1 g), measured (fork length, mm) and the 8 gill arches dissected and mounted on glass slides. Glochidia found on each of the 8 gill arches were counted under a dissection microscope (Leica) at x4 magnification, and glochidia counts for each arch were summed to provide the total glochidia abundance for each fish. Glochidia numbers were counted on two occasions separated several weeks apart to provide data on count repeatability from the same individuals.

**Splenomegaly**

Spleens were dissected from trout hosts at 15 days (n = 27), 31.5 days ( n = 27) and 160 days post exposure (n = 30), weighed (0.001g) and photographed with a Canon EOS D40 fitted with a SIGMA EM-140 DG ringflash and a macro lens (TAMRON SP DI 90 mm 1:2.8, 1:1 magnification), mounted on a copy stand at a fixed 40 cm height from the object. Spleen areas were subsequently digitized from high resolution TIFF images using Image-J (Abramoff et al. 2004) in order to quantify the extent of glochidia-induced splenomegaly (enlargement of the spleen). As with glochidia loads, repeatability in spleen area was calculated from photographs of spleens from the same (matched) individuals measured on two occasions separated several weeks apart.

**Haematocrit determination**

Whole blood from the caudal veins of freshly killed trout (exposed n = 21; unexposed n = 23) was drawn into capillary tubes (75 x 1.5 mm) at 31.5 d.p.e., centrifuged at 3000 *g* for 5 minutes (modified from Woo 1969), and the total packed red blood cell volume read from a haematocrit graduated scale (Hawksley Scientific).

**Ventilation frequency**

Ventilation frequency of trout hosts was estimated from visual measurements of opercular beat rate (OBR; Hawkins et al 2004, 2007; Gibson & Mathis 2006; Brydges et al 2008). A total of 50 exposed brown trout were randomly collected from the EAW hatchery at 160 d.p.e., transported to Swansea University and allowed to acclimatize in a 1 m diameter recirculation tank for 1 week. For OBR measurement, individual fish were placed in 6 three-litre aquaria (25 x 15 x 18 cm) fitted with a constant air supply. A wooden frame and dividers isolated the aquaria and prevented the fish from seeing each other. Small observation holes allowed an observer to view the fish without being seen. OBR was recorded with the aid of a stopwatch at 6 minute intervals during the first hour, then at hourly intervals for 4 hours, before a final reading was taken 24 hours after introducing the fish. This final reading was considered to be the baseline OBR value (Brydges et al 2008).

To examine the response of fish to the threat of predation once the basal OBR had been reached, fish were randomly exposed to either a solution of predator scent or to distilled water (controls), which were introduced remotely to each aquaria via a syringe and aquarium silicone tubing. The predator scent was obtained by homogenising 20 g of spraints from wild otters (*Lutra lutra*) in ten litres of distilled water to obtain a 2 g $l^{-1}$ solution. This solution was strained through a 100 μm mesh and divided into 10 x 1 litre sealable plastic bottles and kept at 4°C until use. OBR was measured one minute after the scent was added, and then every 5 minutes for 30 minutes, after which the fish were removed and killed by an overdose of anaesthesia as above. Aquaria were drained and washed with ethanol to avoid mixing of scents between trials.

**Cryptic colouration**

Whole body photographs of 79 freshly killed trout with varying glochidia loads were taken at 167 d.p.e. with a Canon EOS D40 fitted with a SIGMA EM-140 DG ringflash mounted on a copy stand at a fixed height from the object. High resolution TIFF images were converted to 8-bit greyscale and analysed using Image J software (Abramoff et al. 2004). For each fish, the black colour intensity (darkness value) of a minimum of four parr marks and adjacent flank spaces was calculated along a linear transect extending from the caudal peduncle and continuing along the left flank of the fish (Figure 5.1). This provided an average measurement of the intensity of reflected light from both parr markings and flanks. The difference in reflected light between each parr mark and the adjacent (non-pigmented) flank was then calculated to provide an index of crypsis, on the assumption that vertical parr marking in salmonids increase crypsis (Donnelly & Whoriskey 1993), and therefore the more contrast, the more crypsis.

**Statistical analysis**

General linear models were employed to examine the effect of body size (fork length) and days post-exposure on trout glochidia loads, and tested for glochidia-induced changes in spleen size and haematocrit at various times post-exposure by ANCOVA, using fork length as a covariate. Repeated measures ANOVA was used to compare OBR between treatments, using scent type (blank vs. predator scent) and infection status

(uninfected vs, infected) at 167 d.p.e. as fixed factors and fork length as a covariate to control for variation in body size. For each fish, the beats above basal rate were used in analysis, obtained by subtracting the OBR recording after the fish had been held for 24 hr. from each recording taken following the introduction of the scent. Where Mauchly's test for sphericity could not be met, Greenhouse-Geisser corrected probability values were used. SPSS 16.0, and SYSTAT v. 10 were used for all statistical tests, and applied the logarithmic or square root transformations to improve normality and homogeneity of variances, as required.

## RESULTS

### Variation in glochidia abundance with host body size and days post-exposure

Glochidia counts were reliable, as there were no false negatives (i.e. no encysted fish was overlooked), and repeatability of counts on matched fish host was very high (intraclass-correlation = 0.999, Cronbach's Alpha = 1.000). Glochidia abundance generally decreased with days post-exposure (Table 5.1). Multiple regression ($F_{2,190}$ = 33.927, $P < 0.001$) indicated that variation in glochidia counts (square-root transformed values) was positively associated with body size ($t$ = 2.517, $P$ = 0.013) and negatively associated with days-post exposure ($t$ = -7.703, $P < 0.001$). However, further analysis indicated that the positive effect of body size on glochidia abundance, which was evident at 15 d.p.e. ($F_{1,25}$ = 4.88, $P$ = 0.037) and 31.5 d.p.e ($F_{1,25}$ = 280.02, $P < 0.001$), was not significant at 160 d.p.e. ($F_{1,47}$= 2.404, $P$ = 0.128) or 167 d.p.e. ($F_{1,88}$ = 0.837, $P$ = 0.363), as shown in Figure 5.2.

### Splenomegaly

As with glochidia counts, repeatability of measurements of spleen area was very high (intraclass-correlation = 0.999, Cronbach's Alpha = 1.000). Mean spleen area at 15 d.p.e was 14.3 mm (±7.5), at 31.5 d.p.e. area was 16.5 mm (±6.8) and at 160 d.p.e was 14.1 mm (±7.2). At 15 and 160 d.p.e. glochidia abundance did not have an effect on spleen area; all observed variation could be explained by the host's fork length. However, at 31.5 d.p.e. there was a significant positive effect of glochidia abundance on the spleen area of infected fish ($t$ = + 8.442, $P < 0.001$) when the effect of host body size had been statistically accounted for (multiple regression $F_{2,24}$ = 94.461, $P < 0.001$, Figure 5.3).

### Haematocrit

At 31.5 d.p.e. mean haematocrit values were not related to glochidia abundance ($F_{2,18}$ = 1.959, $P$ = 0.170), nor was there a significant difference in mean haematocrit between exposed (16.42% ±4.47, $n$ = 21) and unexposed fish (16.47% ±3.78, $n$ = 29) when the effect of body size had been accounted for (ANCOVA exposure status $F_{1,41}$ = 0.240, $P$ = 0.627; fork length $F_{1,41}$ = 4.702, $P$ = 0.036)

**Opercular Beat Rate (OBR)**

As data violated the assumption of sphericity (Mauchly's W, $P < 0.001$) the Greenhouse-Geisser correction was applied. OBR was elevated immediately after introducing the fish to each aquaria, and declined over the course of the experiment (RMANOVA $F_{1,14} = 6.558$, $P < 0.001$; Figure 5.4). The final OBR reading at 24 hr. was considered to be an accurate measure of the baseline ventilation rate. Following the addition of scented or distilled water, OBR significantly increased in the presence of predator scent but not in the presence of blank water ($F_{1,48} = 244.217$, $P < 0.001$; Figure 5.5). Overall, OBR was significantly related to fork length ($F_{1,45} = 6.906$, $P = 0.012$) but not to infection status ($F_{1,45} = 0.920$, $P = 0.343$) or to glochidia abundance ($F_{1,45} = 2.080$, $P = 0.156$).

**Cryptic colouration**

As the total glochidia counts were not normally distributed (one sample Kolmogrov-Smirnov test, $P < 0.001$) data were log-transformed before multiple regression. Glochidia abundance was not a significant predictor of either contrast ($t = -1.236$, $P = 0.220$) or intensity of parr markings ($t = -1.487$, $P = 0.141$) when the effect of body size had been statistically controlled for.

## DISCUSSION

The results of this study, based on two different cohorts of juvenile (0+) brown trout, broadly supports the conclusion that glochidia abundance is positively correlated with host body size, but that this effect is transitory and restricted to the initial stages of encystment. A positive association between glochidia abundance and host body size has already been noted in salmonids (Bauer & Vogel 1987). Thus, Young & Williams (1984) observed that larger trout had a greater abundance of glochidia than smaller trout, and similarly, Hastie & Young (2001) reported that larger 0+ salmon initially had a significantly greater abundance of glochidia than smaller conspecifics, but that this became non-significant over time. However, both Cunjack & McGladdery (1991) working in Nova Scotia with 0+ wild salmon, and Beasley (1996) working in Ireland with wild trout and salmon (of unknown age) found that there was no association between glochidia load and host size. In contrast, other authors have found glochidia prevalence to be significantly lower in larger fish, and that larger fish harboured relatively fewer glochidia than smaller ones (Bauer 1987b). However, many of the earlier studies did not discriminate between different age classes (i.e. 0+, 1+, etc..) of the fish hosts, which were segregated by body size alone. Therefore, it is likely that some of these contradictory effects are probably due to acquired immunity caused by previous exposure of older fish, rather than by a genuine effect of host body size. In contrast, the positive relation between glochidia abundance and host body size found in our study is based on fish of the same age (0+), which had never been in contact with mussel glochidia, and which could not, therefore, have developed acquired immunity.

In many host-parasite systems, splenomegaly can result from an immunological host response to antigenic material (Contamin et al 2000; Morand & Poulin 2000; Brown & Brown 2002; Stanley & Engwerda 2007; Cowan et al 2009). In this study, splenomegaly was only observed after one month post exposure, when it was positively related to glochidia abundance. But again, this effect appears to be transitory, as no evidence of spleen enlargement was found before or after that period. This suggests that in naïve fish the anti-glochidial immune response probably takes several weeks to develop, and that the fish host quickly recovers. Humoral and tissue reactions to *M. margaritifera* glochidia have been described in brown trout (Bauer 1987b; Bauer &

Vogel 1987), and also in coho salmon (*Oncorhynchus kisutch*) encysted with *M. falcata* glochidia (Fustish & Millemann 1978). In the bluegill sunfish, *Lepomis macrochirus*, a humoral and mucosal antibody response against glochidial antigens of *Utterbackia imbecillis* was found at 20 and 60 days post exposure (Rogers-Lowery et al 2007). In previously challenged fish, anti-glochidial antibodies have been identified in host blood much sooner following a repeated glochidial challenge, indicating the existence of acquired immunity. For example, Bauer & Vogel (1987) note the production of *M. margaritifera*-specific anti-glochidial antibodies in previously challenged brown trout as early as 7 days post exposure. The same results were obtained by O'Connell & Neves (1999), who detected anti-glochidia antibodies in previously exposed *Ambloplites rupestris* 7 days after a repeated challenge by glochidia of *Villosa iris*.

No significant effect of glochiodosis on the haematocrit value of trout blood was found at one month post-exposure, although the sample size was admittedly small and the method employed to determine haematocrit crude. In a previous study glochidia abundance was also found to be unrelated to salmonid host condition or plasma lactate levels (Treasurer et al 2006). However, plasma chloride levels in glochidia-encysted juvenile salmon were found to be significantly higher 10 days after sea transfer (Treasurer & Turnbull 2000), suggesting that glochidiosis may affect the ability of salmon to adapt to the marine environment.

Gill parasites such as unionid glochidia may be expected to have an impact on the host's respiration performance, as seen by the elevated ventilation frequency of *Micropterus salmoides* infected by glochidia of *Lampsilis reeveiana*, even several months post glochidial excystment (Kaiser 2005). Therefore, opercular beat rate may be expected to be elevated among encysted fish due to impaired gas exchange resulting from cyst-forming gill parasites. Yet, ventilation frequency was unrelated to glochidia loads in this study, when the effect of body size was statistically accounted for at 161 days pots-exposure. Moreover, compared to uninfected controls, no increase in ventilation rate was observed amongst encysted trout. It can thus be concluded that, within the range of glochidia loads found in this study (1-204 glochidia per fish), glochidia encystment appears to have no detectable effect on host respiration performance. The only previous study to find an effect on host respiratory performance

in relation to glochiodosis had an average of 632 glochidia per fish, compared to 37 glochidia/fish in our study (Kaiser 2005). Thus, it is not known if higher glochidia loads would have impaired the respiratory performance of brown trout, or if as with splenomegaly or body size, the effect is perhaps also transitory and restricted to the initial stages of encystment. We also failed to find any evidence for glochidia-induced changes in ventilation rates when we exposed encysted and control hosts to the scent of a known trout predator. As expected, trout reacted by increasing their ventilation frequency compared to fish exposed to blank water, but this effect was unrelated to glochidia loads. It may be that fish respond to the predator scent by elevating their ventilation rate, regardless of glochidia abundance, such is the strength of the antipredatory response amongst salmonid fish (Kats & Dill 1998). It may be worthwhile repeating this study at an earlier phase during encystment, when the immune response appears to peak and glochidia loads are generally higher.

Unlike trophically-transmitted parasites that can disrupt host crypsis and make the intermediate host more vulnerable to predation (Ness & Foster 1999; Barber et al 2000; Moore 2002), glochidia encystment in this study did not appear to disrupt the cryptic colouration of brown trout. As glochidia are not trophically transmitted and depend on the host survival for their own survival, it appears advantageous for glochidia not to make the host more vulnerable to predation, a common strategy seen amongst trophically transmitted parasites. For example, the trematode *Leucochlordium* alters the colouration of its intermediate snail host to make it more conspicuous (Moore 2002), while the trematode *Diplostomum spathaceum* forms cataracts in the eyes of rainbow trout that impair the host's ability to regulate its cryptic colouration, making it more visible and vulnerable to the parasites' avian definitive predatory host (Seppala et al 2005). Our study shows that brown trout encysted with the glochidia of *Margaritifera margaritifera* do not suffer from impaired crypsis, as can occur in other host-parasite relationships (Moore 2002).

The relationship between unionid glochidia and their various hosts is not clear; whilst perhaps not truly pathogenic, the symbiosis-protocooperation explanation (Geist 2010), or the phoretic description of this relationship (Barnhart et al 2008) do not fully explain all the observed effects of glochidiosis. The transitory spleen enlargement

observed in this study, along with the observed temporal changes in the effect of host body size on glochidia abundance, and the acquired immunity reported by others, strongly suggest that the impacts of glochidia on the hosts are slight. Results for *M. margaritifera* and other freshwater mussels (Fustish & Millemann 1978; Bauer 1987b; Bauer & Vogel 1987; O'Connell & Neves 1999; Rogers-Lowery et al 2007) suggest that there is an advantage to be gained from shedding glochidia, at least during the initial period of encystment, thereby providing a strong argument against the relationship being phoretic or a form of symbiosis-protocooperation. However, our study - as well as that of Treasurer et al (2006), also suggest that glochidia have little or no impact on the hosts' haematological condition, or on its respiratory performance - at least within the range of glochidia numbers commonly seen in the wild. These results, along with the lack of crypsis breakdown commonly seen in other, tropically-transmitted, true parasites, lend weight to the theory that the glochidia of *M. margaritifera* have only a transitory effect on the salmonid hosts' physiology, and do not overly impact host fitness.

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Table 5.1. Variation in glochidia prevalence and abundance in 0+ brown trout hosts sampled at various days post-exposure (d.p.e)

| Sampling year | Spat release | D.P.E | No. trout examined | FL (mm) (SE) | Prevalence (%) | Mean glochidia/fish (SE) |
|---|---|---|---|---|---|---|
| 2008 | 22/09 | 15 | 27 | 91.3 (±3.7) | 100.0 | 100.7 (±18.6) |
| | | 31.5 | 27 | 98.4 (±2.7) | 100.0 | 150.9 (±2.9) |
| | | 160 | 49 | 107.3 (±3.5) | 55.0 | 36.7 (±7.9) |
| 2009 | 28/09 | 167 | 90 | 174.4 (±1.9) | 47.7 | 54.1 (±7.6) |

**Fig. 5.1**

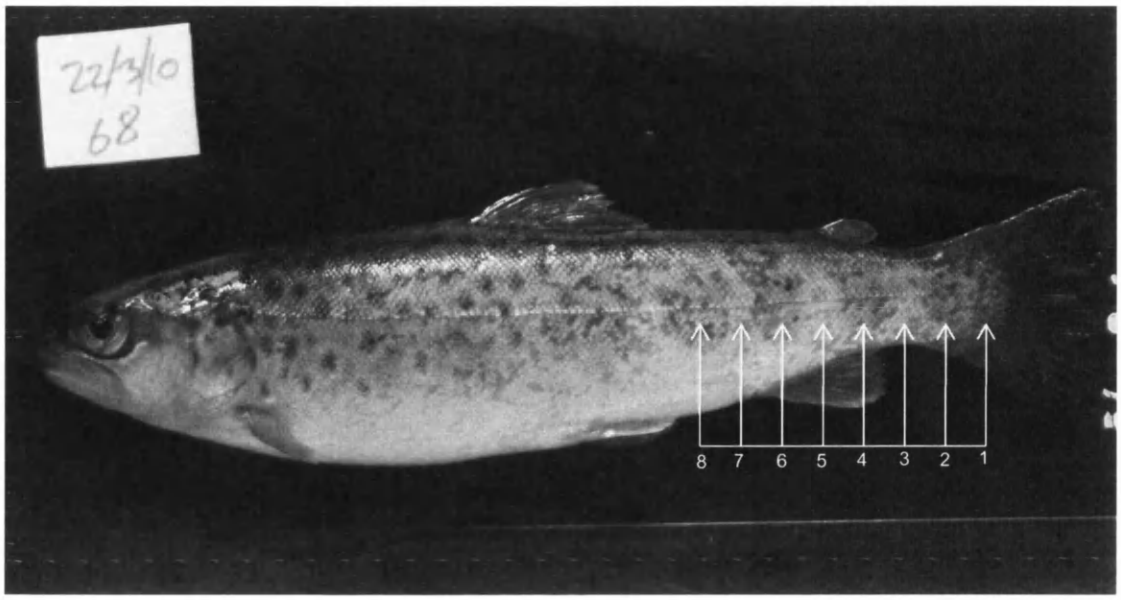


Figure 5.1. Location of measurements of colour intensity in parr parks and adjacent flanks for analysis of crypsis.

**Fig. 5.2**

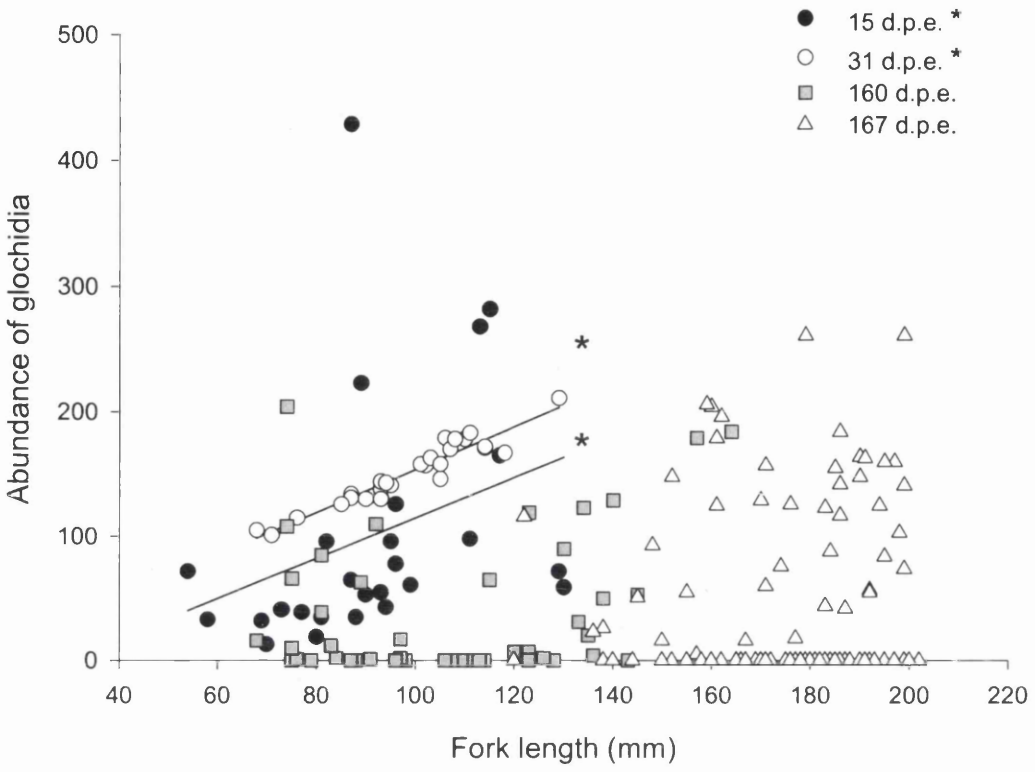


Figure 5.2. Relationship between glochidia abundance and fork length of brown trout hosts over the course of encystment. * denotes a significant positive relationship.

**Fig. 5.3**

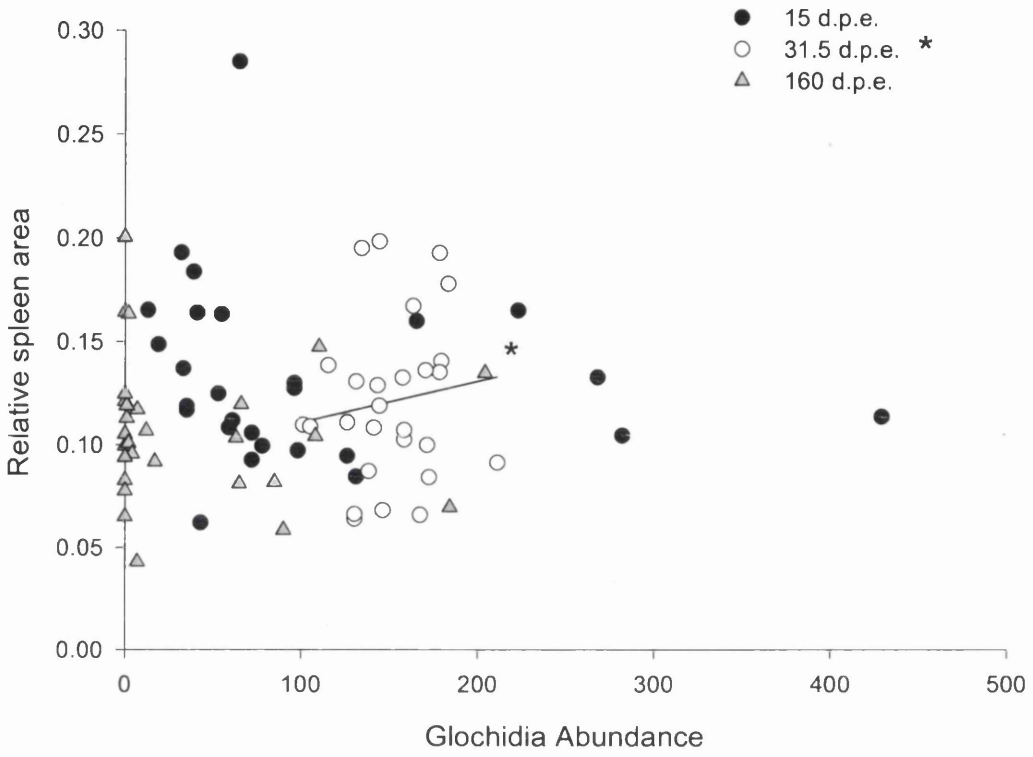


Figure 5.3. Relationship between glochidia abundance and relative spleen area of brown trout hosts over the course of encystment. * denotes a significant positive relationship.

**Fig. 5.4**

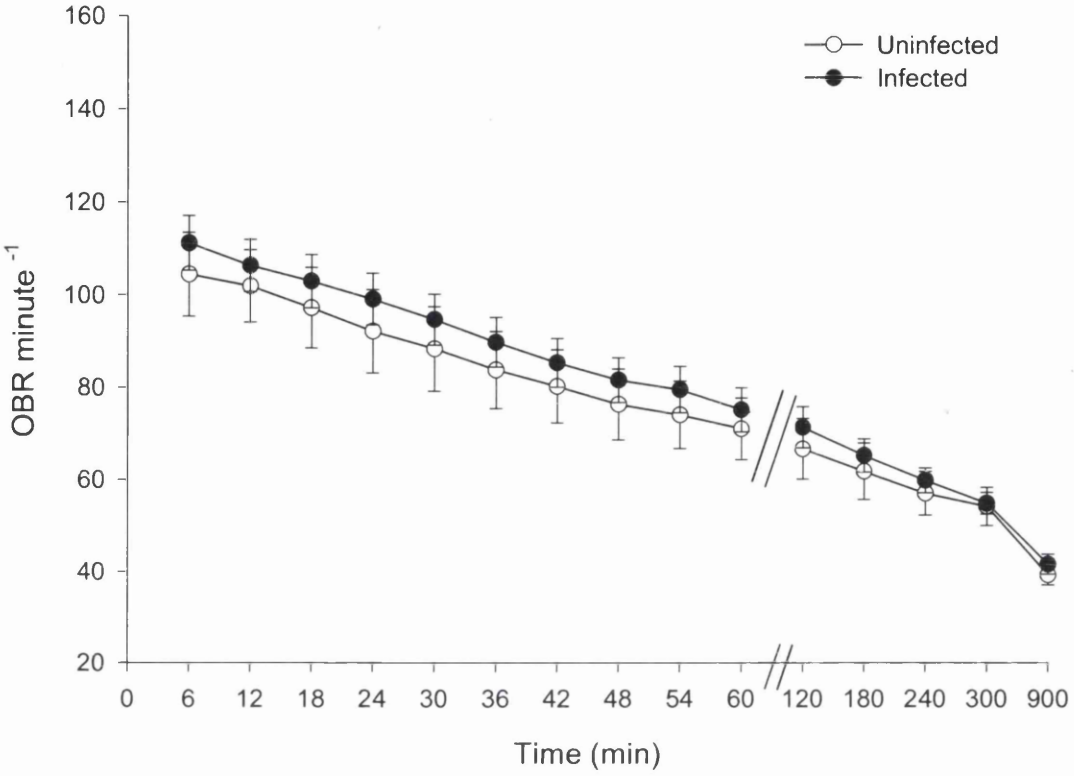


Figure 5.4. Temporal variation in opercular beat rate (mean ±95 CI's) of uninfected and glochidia-infected juvenile brown trout over 24 hours.

**Fig. 5.5**

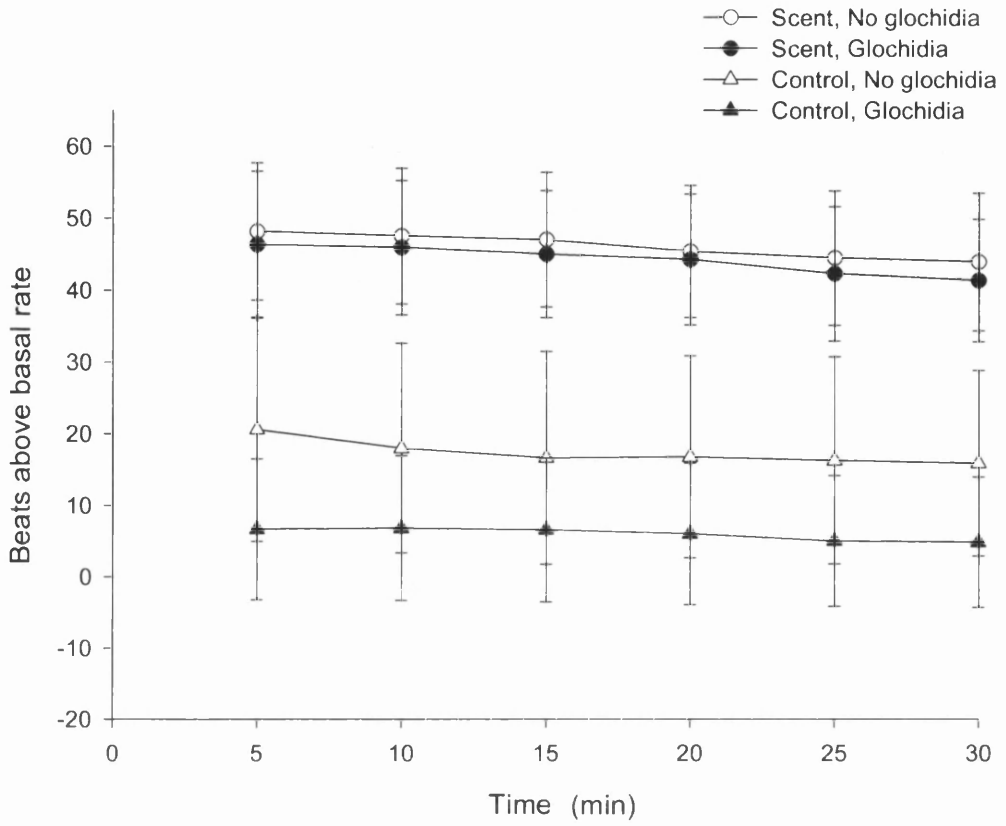


Figure 5.5. Temporal variation in opercular beat rate above basal levels (mean ±95 CI's) of uninfected and glochidia-infected juvenile brown trout exposed to blank water (controls) or to predator-scented water.

# Chapter VI.

## Backseat driving: behavioural effects of *Margaritifera margaritifera* on brown trout (*Salmo trutta*)

# Chapter VI.

# Backseat driving: behavioural effects of *Margaritifera margaritifera* on brown trout (*Salmo trutta*)


## ABSTRACT

Trophically-transmitted parasites can alter the behaviour of their intermediate hosts to make them more vulnerable to predation, thereby facilitating their own transmission. However, the effect of non-trophically transmitted parasites that depend on the survival of their hosts for their own survival has seldom been examined. Here the risk-taking behaviour and predator avoidance of juvenile brown trout encysted with glochidia of the freshwater pearl mussel *Margaritifera margaritifera* at several times post-encystment was examined. Latency to emerge from a hide, a proxy for boldness and risk-taking behaviour, was positively related to glochidia loads at all times post-encystment, and was significantly lower among encysted trout than among unexposed, control fish. The scent of a sympatric mammalian predator (*Lutra lutra*) in the water significantly decreased risk-taking behaviour and induced spatial avoidance in brown trout, regardless of glochidia abundance or infection status. Results indicate that the freshwater pearl mussel does not impair predator recognition or spatial avoidance of its host, whilst potentially increasing host survival by making it more risk-averse, thereby limiting contact with predators.



**Keywords:** brown trout, behaviour, glochidia, latency, glochidia, *Margaritifera margaritifera*

## INTRODUCTION

Whilst trophically-transmitted parasites are capable of altering fish responses to predators in order to facilitate their own transmission (e.g. Barber et al 2000; Moore 2002; Mikheev et al 2010), the effects of non-trophically transmitted parasites on fish behaviour remain largely unknown. Behaviour represents one of the most important determinants of fish survival (Griffin et al 2000; Biro et al 2004; Hawkins et al 2008), and can therefore be expected to be under strong selective pressure. Salmonids are obligate, definitive hosts of the glochidia (the infective larval stage) of the endangered freshwater pearl mussel *Margaritifera margaritifera*, therefore trophic transmission is not necessary. Yet, very little is known about the effects of glochidia on salmonid behaviour, despite the fact such interactions may underpin the survival of both host and parasite on this fish-mussel system (Thomas et al 2010). Freshwater mussel glochidia must remain attached to their host for varying periods of time in order to complete their development. As obligate parasites, the fate of encysted glochidia is inexorably linked to that of the host; if during the course of encystment the fish dies, then so do glochidia. It is hypothesised that glochidia would not impair the survival of its host; in fact it can be argued that if glochidia were to have an effect on host behaviour, it would be to reduce the likelihood of predation or death. Indeed, the relationship between *M. margaritifera* and its salmonid hosts has been suggested to be an example of symbiosis-protocooperation (Ziuganov & Nezlin 1988; Geist 2010), though no studies have experimentally tested this hypothesis.

Latency to emerge from a hide constitutes a useful proxy for risk-taking behaviour along the boldness-shyness continuum (Wilson et al 1994; Brown et al 2005) which correlates well with boldness (Sneddon 2003, Wilson & Stevens 2005), foraging success (Wilson et al. 1993; Wilson & Godin 2009), and anti-predatory behaviour (Sundstrom et al 2005; Brown et al 2007). It can be used to assess the willingness of an organism to investigate a novel habitat under various threats of predation. Similarly, the ability of prey to detect and react to the presence of potential predators can be examined relatively easily through spatial avoidance tests (e.g. Vilhunen & Hirvonen 2003), as the threat of predation is a powerful selective agent (Mirza & Chivers 2000; Brown 2003). Prey can detect predators innately or through acquired experience (Ferrari et al 2010),

often facilitated in the aquatic environment by the recognition of specialised alarm cues released by conspecifics when they are injured or digested by predators. This makes it possible to use chemical cues, rather than predators themselves, to test for anti-predatory behaviours.

Here the willingness to take risks and the anti-predatory behaviour of juvenile brown trout encysted with glochidia of the freshwater pearl mussel at various times post-encystment was tested. The null hypothesis was that, unlike tropically transmitted parasites, the encysted glochidia of the freshwater pearl mussel would make its host less willing to take risks (more risk-averse), and more likely to recognize and react to predators, if that served to increase the host's, and therefore also the parasite's survival.

## METHODS

### Study populations

Hatchery-reared juvenile 0+ brown trout, *Salmo trutta,* from the Rivers Usk and Mawddach stocks were exposed to glochidia of *M. margaritifera* each autumn during 2007, 2008 and 2009 at the Cynrig and Mawddach hatcheries as part of the Environment Agency (Wales) *ex situ* conservation programme for the freshwater pearl mussel. Days post exposure (d.p.e.) were calculated as a mid point between the date of the last negative sampling occasion (when no glochidia were found) and the first positive sampling occasion when glochidia were found. The range between negative and positive sampling events was 15 - 27 days for the three sampling periods.

### Behavioural Assays

Behavioural assays were conducted at the Freshwater Research Unit (FRU), Swansea University.

*Latency to leave shelter*

Latency to emerge from a shelter was determined for three cohorts of 0+ *S. trutta* tested at 31.5 (n = 60), 140 (n = 48), and 167 days post-exposure (n = 30) using methods adapted from Brown et al (2005) and Burns (2008) (Table 6.1). Fish were netted individually out of a holding tank and transferred into a covered shelter (16cm L x 39cm W x 16cm D) in one of two flow-through hatchery troughs (280cm L x 40cm W x 16cm D; average flow 21 ± 1 L $min^{-1}$) and allowed to acclimatize for 15 minutes, a period shown to be adequate for studies of anti-predatory behaviour in other salmonids (Vilhunen and Hirvonen, 2003). Following this acclimatization period, an observer hidden behind a screen would raise a drawbridge by means of a pulley, allowing fish access to the remainder of the trough for a further 15 minutes. The time each fish took to emerge from the shelter (whole body) into the novel habitat was recorded with a stopwatch by the hidden observer to provide the latency (*L*) in seconds. As in most studies of boldness (e.g. Brown et al., 2005) we assigned a maximum ceiling value (900 seconds in our case) to those fish that did not come out of the hide.

*Predator Avoidance*

Predator avoidance was examined on 90 0+ brown tout trout at 167 days post-exposure. Fish were transported to FRU from the Cynrig hatchery and allowed to acclimatise for 1 week. Trials were conducted in a flow-through hatchery trough (280cm L x 40cm W x 16cm D) modified to serve as a Y-maze (Vilhunen & Hirvonen 2003, Figure 6.1). For this, the trough was divided by a central partition into two identical channels, each fitted with a submerged spray bar at the inlet and a common water supply from a carbon-filtered, dechlorinated source. Average flow was 19 L $min^{-1}$ (± 0.6 L) and surface velocities varied between 10 and 13 cm $sec^{-1}$ in each arm.

Fish were netted out of a holding tank and into a covered shelter (16cm L x 39cm W x 16cm D) at the outlet, and allowed to acclimatise for 15 minutes. Following the acclimatisation period, the gate was raised remotely and fish were free to choose one of the two channels, which were scented with either blank water (control) or a solution of predator scent. A fully factorial design ensured that the scented arm was randomly chosen and equally represented in both left and right arms. Predator scent prepared for a previous experiment (Chapter V) was frozen and thawed before use for this experiment. Briefly, 20 g of spraints from wild otters (*Lutra lutra*) were homogenised in ten litres of distilled water to obtain a 2 g $l^{-1}$ solution. This solution was strained through a 100 µm mesh and divided into 10 x 1 litre sealable plastic bottles, frozen then defrosted at 4°C before use. Blank (distilled) water and solutions of predator scent were administered via separate 1 L drip bags mounted at upstream end of each arm of the trough, and hidden from view behind a screen to minimise disturbance. One minute before the gate of the shelter was raised, the valves of the drip bags were opened and 4.3 ml of solution was added to the water over the 15 minute trial, at a rate of approximately 0.004 ml $sec^{-1}$. The latency to emerge from the hide was recorded by a hidden observer as per above, and the time fish spent in both scented and control arms was recorded on separate stopwatches to give a measure of spatial avoidance.

**Data Analysis**

Following the behavioural assays, fish were rapidly killed by an overdose of anaesthetic, measured (fork length, mm), weighed (wet weight, 0.1 g), and the gill arches dissected

and mounted on glass slides. Glochidia were counted on each gill arch by light microscopy (Leica) at x4 magnification. Latency was log-transformed to improve normality and homogeneity of variances. The time spent in scented and control arms was calculated as a proportion of total time spent in the trough (excluding time spent in the hide). General linear models were used in SPSS 16.0 and Systat 10 to examine variation in either latency or proportion of time in scented arm as a function of total glochidia abundance, number of days post-exposure and fork length as predictors. Data were square-root transformed (latency) or arcsine transformed (proportion of time spent in arm) to meet normality and homogeneity of variances, as needed.

## RESULTS

### Latency to leave shelter

When tested with blank water, trout with encysted glochidia took significantly longer to emerge from the shelter than uninfected hosts at all times post-exposure (Figure 6.2). Thus, mean latency to emerge from the hide was 273 sec. (at 31.5 dpe), 505 sec. (140 dpe) and 380 sec. (167 dpe) for control (uninfected) fish compared to 577, 563 and 497 sec. for encysted fish over the same times post-encystment, respectively. Multiple regression ($F_{3,134}$ = 5.238, $P$ = 0.002) indicated that latency to emerge from the shelter was positively related to glochidia abundance ($t$ = 3.532, $P$ = 0.001), but not to host body size ($t$ = -0.591, $P$ = 0.556) or number of days post-exposure ($t$ = 1.212, $P$ = 0.228). However, unlike under control conditions, in the presence of predatory scent, latency to leave shelter was unrelated to the number of glochidia harboured by the host ($t$ = 1.703, $P$ = 0.095), or its body size ($t$ = -0.891, $P$ = 0.377; $F_{2,50}$ = 1.707, $P$ = 0.192) at 167 days-post-exposure.

### Predator Avoidance

We found no significant difference in the proportion of time spent in each arm of the Y-maze when fish (n = 30) were exposed to blank water only (paired t-test $t_{27}$ = 1.485, $p$ = 0.481), indicating that the test arena had no inherent bias for either arm. The proportion of time spent by trout in the arm scented with predator cues (mean = 0.312) was significantly less than the 0.5 expectation with blank water ($F_{1,86,}$ = 17.33, $P$ < 0.001), but this was unrelated to infection status (Figure 6.3). There was no significant difference between infected and uninfected trout in the proportion of time spent in the scented arm ($t_{51}$ = -0.366, $P$ = 0.716), indicating that glochidia encystment did not alter predator avoidance behaviour. This point was confirmed by multiple regression ($F_{2,50}$ = 0.125, $P$ = 0.883), which indicated that predator avoidance was unaffected by glochidia loads ($t$ = -0.126, $P$ = 0.900) or body size ($t$ = -0.468, $P$ = 0.642).

## DISCUSSION

Trophically transmitted parasites are known to be able to manipulate the behaviour, physiology and morphology of their intermediate hosts in order to increase the likelihood of predation by the definitive host, therefore facilitating their own transmission (Barber et al 2000; Moore 2002; Bass & Weis 2009). In contrast, the behavioural responses of fish to non-trophically transmitted parasites, such as salmonids to glochiodosis, has seldom been studied, despite the overarching influence of behavioural traits for fitness, including foraging (Brown et al 2003; Orlov et al 2006) and predator avoidance (Sundstrom et al 2005). Latency to emerge from a hide has been found to represent a reliable measure of risk taking behaviour in fish (Wilson et al 1993; Sneddon 2003; Wilson & Stevens 2005; Wilson & Godin 2009), and our results suggest that, when tested with blank water, glochiodosis makes juvenile brown trout less bold (more risk-averse), regardless of body size or time since encystment. By making the host more risk-averse, glochidia can potentially reduce the exposure of host to predators, thereby maximizing its own survival. However, there may be a trade-off, as risk-averse fish may not be able to forage as efficiently, or be as successful in establishing a territory, as other individuals (Coleman & Wilson 1998).

The mechanisms that may enable glochidia to alter the risk-taking behaviour of its definite trout hosts are uncertain, and can only be speculated upon. Some parasites can directly damage or manipulate the host central nervous system by releasing neurotransmitters and neuromodulators, thereby interfering with 'normal' expressions of behaviour (Lafferty & Morris 1996; Barber & Wright 2005). Other parasites affect the host's nutritional status, and can thus indirectly alter behaviours by changing the motivation for tasks such as foraging (Milinski 1990; Cunningham et al 1994; Ranta 1995). In these ways, numerous trophically-transmitted parasites, especially helminths, influence the behaviour of their intermediate hosts and facilitate their own transmission (reviewed by Poulin 1994). In the case of *M. margaritifera* glochidia, salmonids are the definitive host, and as such any effects on host behaviour is not expected to facilitate predation of the host; rather it is assumed that they would be aimed at ensuring the hosts' survival.

A second, potential explanation for the increased latency observed amongst infected trout hosts may lie on the physiological impact that gill-encysted glochidia may have on the hosts' respiratory system. Glochidia of other freshwater mussels have been found to impair the effectiveness of gas exchange in fish, at least at high glochidia loads (Kaiser 2005). Altered behaviour as a result of physiological stress and morbidity ("ill health") is known to occur in other species (King et al 2001; Fenwick 2009; D'Acremont et al 2010), and morbidity-induced behavioural changes could therefore also result from the physiological impact of glochidia encystment on gill tissue.

Whatever the mechanism by which glochidia influence host behaviour, elevated latency during encystment can result in reduced foraging success, but also in reduced threat of predation (Lima & Dill 1990). Reduced foraging ability would lead to a greater risk of malnutrition and starvation (Brown et al 2003; Orlov et al 2006), especially for fish with encysted glochidia, as the splenomegaly observed at 31.5 days post exposure in a previous study (Chapter IV) is inferred to place additional demands on the hosts energy reserves. Despite this risk of malnutrition or starvation, by being less willing to investigate novel habitats glochidia-encysted fish would theoretically be at less risk of predation, resulting in lower rates of predation over the course of encystment.

In addition to being a measure of risk-taking behaviour, latency can also be a measure of how animals react to predators or other stimuli in novel situations (Reale et al 2000; Brown & Braithwaite 2004; Brown et al 2005; Wakeling 2006; Dadda et al 2010). Brown trout juveniles will change their behaviour and habitat preferences when confronted with the threat of predation (Greenberg et al. 1997; Vehanen & Hamari 2004). In the present study, juvenile brown trout strongly avoided the water scented with chemical cues from a predator, presumably in order to reduce the perceived threat of predation, much in the same way as other fish do (e.g. Ferrari et al 2007). In the presence of predator cues, elevated latency has been observed in other prey species such as the bishopfish *Brachyraphis episcopi* (Brown et al 2007) and Atlantic salmon (Roberts 2010). Regardless of glochidia abundance, brown trout displayed elevated latency in the presence of a predator scent, increasing the time to emerge from a hide. As such, glochidia conferered no disadvantage to brown trout with regards to predator avoidance behaviour, but it did not enhance it either. The fitness trade-offs of remaining

in shelter or fleeing once fish have detected the scent of a predator remain unclear (Brown et al 2005, 2007). What is clear is that, in the absence of chemical stimuli that signal immediate danger, emerging from a hide into a novel environment exposes an organism to an element of risk, which is counterbalanced by the benefit obtained from foraging for food. Captive-reared brown trout are less likely to use shelters when confronted with a predator than wild counterparts (Alvarez & Nicieza 2003) and tend to be maladapted to foraging for live food, resulting in high mortality and low growth rates (Brown et al 2003; Orlov et al 2006). Glochidia do not appear to influence the innate ability of brown trout to detect and avoid predators, yet may influence survival by limiting the contact between fish and predators during the initial stages of encystment.

There is a clear evolutionary advantage to be gained by a parasite from facilitating transmission rate and transmission success (Moore 2002) and this can be achieved by altering host behaviour. For example, the trematode *Diplostomum spathaceum* can alter the aggressiveness (Mikheev et al 2010) and shoaling behaviour (Seppala et al 2008) of rainbow trout, thus making the host fish more vulnerable to predation by the parasites' definitive host. According to the Basic Model of May and Anderson (1990), under constant conditions increased parasite transmission rate will lead to an increased reproductive ratio, thereby providing the evolutionary rationale behind attempting to alter host behaviour.

Mortality during the early stages of the freshwater pearl mussels' lifecycle is very high, and even small changes in survival rates could have profound impacts on recruitment due to the high reproductive output of this species (Hastie & Young 2003). Hence, there is an evolutionary advantage to be gained by glochidia from ensuring the survival of the host. Recent studies (Geist et al 2006; Osterling et al 2008) suggest that host fish density has little impact on juvenile mussel recruitment rates. One possible explanation for this may be that - by influencing survival rates of infected fish - glochidia are not reliant on a large number of hosts, but rather on the survival of a few highly infected individuals. Our results suggest that far from being passive passengers on their fish host, the glochidia of *Margaritifera margaritifera* can influence host risk-taking behaviour, without impairing its anti-predatory behaviour. Such changes hold the

potential to enhance host survival, which would in turn facilitate the survival of the mussel into the post-parasitic stage.

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Table 6.1. Variation in the body size, days post-encystment (d.p.e.) and glochidia abundance of juvenile 0+ brown trout used in the behavioural assays.

| D.P.E | FL (mm) | Glochidia Prevalence (%) | Mean Glochidia/fish (SD) |
|---|---|---|---|
| 31.5 | 99.2 (±14.0) | 100 | 153.4 (±26.3) |
| 140 | 195.2 (±25.4) | 39.6 | 382.5 (±407.3) |
| 167 | 174.4 (±18.7) | 47.7 | 54.1 (±72.6) |

**Fig. 6.1.**

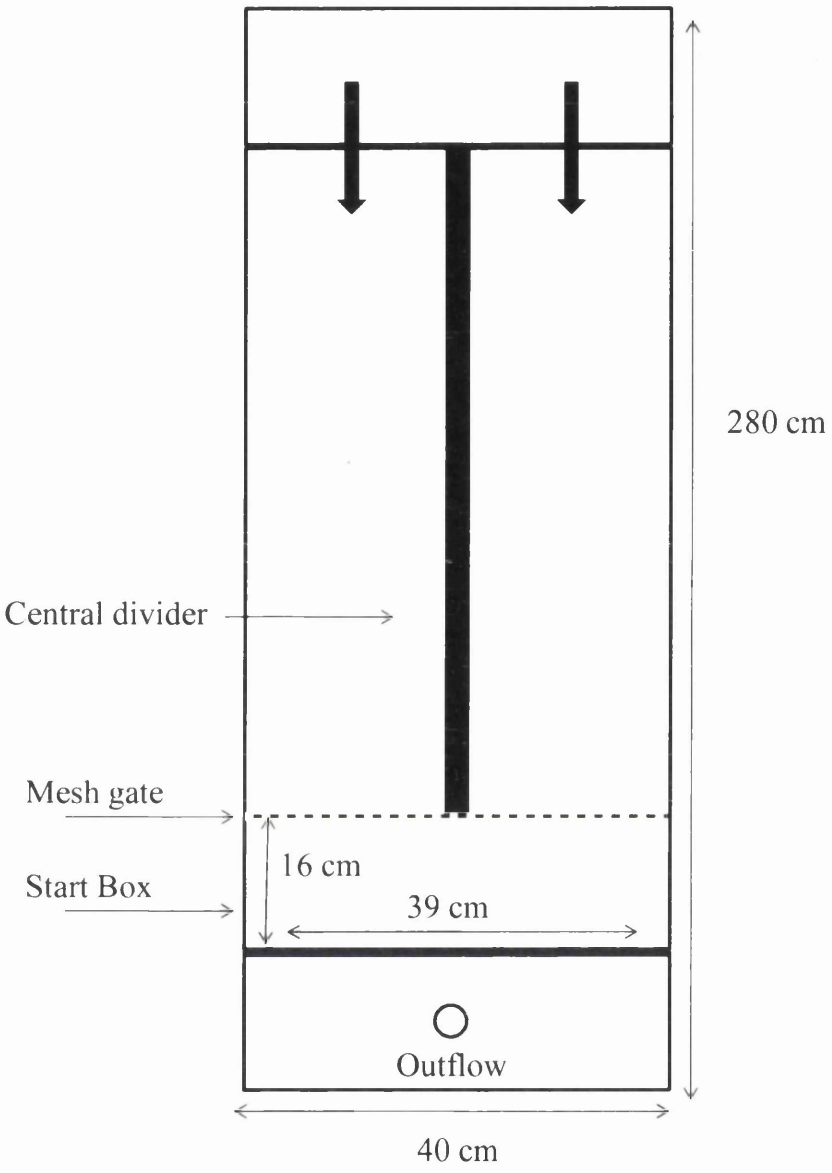


Figure 6.1. A diagram of the Y-maze employed for testing the predator avoidance of brown trout. Blank and scented waters are kept separate by the central divider, whilst fish are able to choose which arm to occupy once the mesh gate to the start box has been opened.

**Fig. 6.2.**

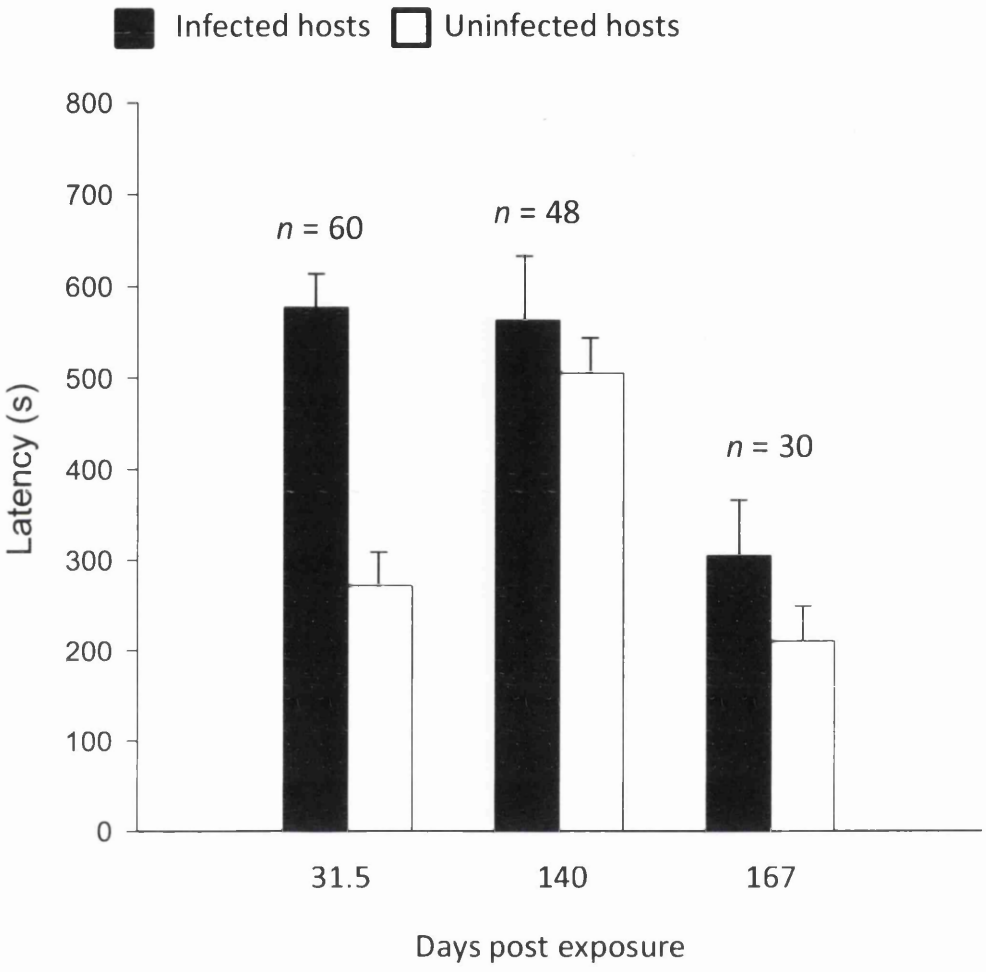


Figure 6.2. Mean latency (sec) to emerge from a hide among glochidia-encysted and un-infected juvenile brown trout at 31.5 days post exposure ($n$ = 60), 140 d.p.e. ($n$ = 48) and 167 d.p.e. ($n$ = 30). Bars represent 1 SE.

**Fig. 6.3.**

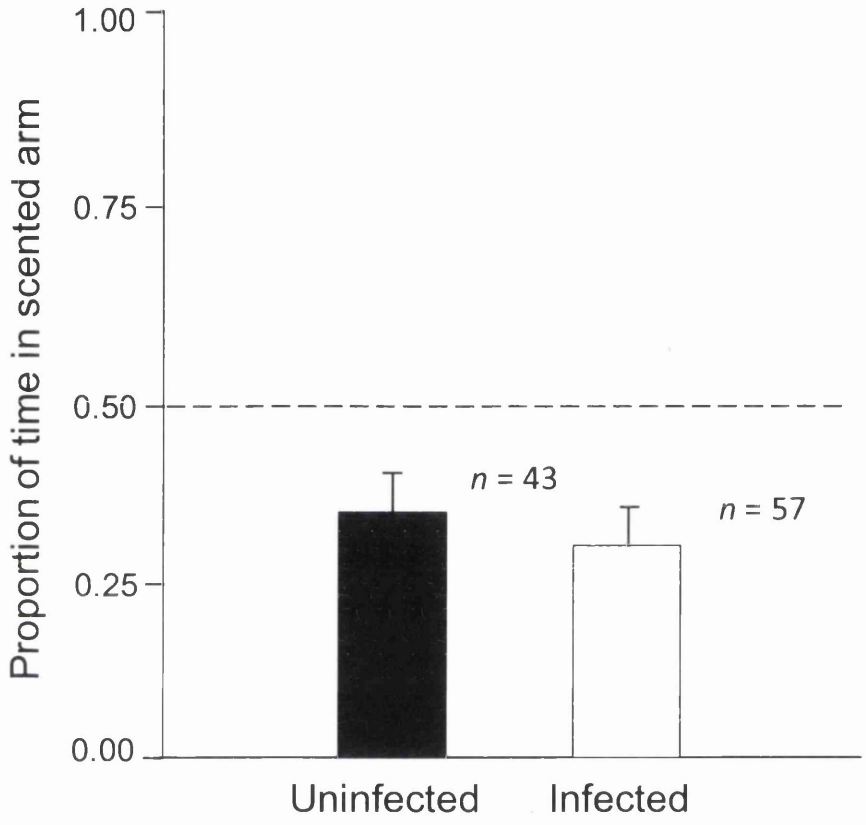


Figure 6.3. Mean proportion of time spent in the predator-scented arm of the Y-maze vs. the 50% random expectation (dotted line) among glochidia-encysted ($n$ = 43) and uninfected ($n$ = 57) juvenile brown trout at 167 days post-exposure. Bars represent 1 SE

# Conclusions

1. Many species of freshwater mussels are threatened with extinction throughout their range and rank amongst the most endangered aquatic organisms in the world. Reasons for freshwater mussel declines are numerous, but can in nearly all cases be traced to human activities resulting in habitat degradation, pollution, overfishing, host declines, and loss of river connectivity. The freshwater pearl mussel *Margaritifera margaritifera* has suffered a particularly marked range contraction and decline in abundance over the last 100 years, but conservation efforts have tended to be hampered by limited knowledge and understanding of key life stages, and little or no monitoring of results. As with many other endangered species, its conservation has been attempted through both *ex situ* and *in situ* approaches, with varying measures of success.

(a) *Ex-situ* methods have been, by far, the preferred approach, despite the fact that there have been few, if any, successful mussel reintroductions into the wild. Lessons from other *ex-situ* conservation programmes, for example those involving salmonids, have stressed the inherent risks of captive breeding. The effects of domestication, the over-representation of particular alleles, and poor survival of hatchery-reared organisms compared to wild counterparts, are all inherent problems of captive breeding (e.g. Snyder et al 1996). In this thesis, I argued that no matter how much effort is directed to captive breeding, unless the underlying threats are not first identified and addressed at meaningful spatial scales (i.e. whole catchments), freshwater mussels will likely continue to decline.

(b) *In-situ* conservation methods are more likely to be successful in the long term, but there are relatively few published studies regarding the benefits of habitat remediation techniques for freshwater mussels. Instead, practitioners have tended to adapt existing salmonid *in-situ* conservation techniques. Most *in-situ* efforts have been uncoordinated, disjointed, and rarely published, and this has made it very difficult to learn from successes and failures. There is an urgent requirement for more empirical research on

the effectiveness of habitat improvement and an evaluation of the successes (and failures) of such methods. An integrative approach that combines habitat restoration with *ex-situ* breeding is likely to be most successful option.

2. Novel technologies, such as the Hall-effect magnetic sensors used in this thesis, can help to uncover complex aspects of bivalve behaviour, and these can used to assess welfare of adult mussels in captivity and to quantify the impact of likely stressors such as siltation, eutrophication, and pollution. Such technologies are largely unobtrusive, do not seem to adversely affect mussels, and can provide important information on activity patterns, including foraging rhythms and timing of spatting.

3. Direct exposure host specificity studies indicate that, in addition to the known hosts' brown trout (*Salmo trutta*) and Atlantic salmon (*Salmo salar*), *M. margaritifera* can also successfully encyst on the gills of arctic charr (*Salvelinus alpinus*). However, salmonid hosts differed significantly in glochidia prevalence and abundance, as well as in their response to glochiodosis. Brown trout had the highest glochidia abundance and the most developed cysts, followed by arctic charr which had intermediate glochidia loads but high prevalence, and Atlantic salmon which was only rarely encysted. Thus, the suitability of salmonids as hosts for the freshwater pearl mussel seems to adhere to predictions of models of host-parasite dispersal and co-evolution, being highest for the host with the lowest dispersal (brown trout), intermediate for the partially migratory arctic charr, and lowest for the anadromous, and highly migratory Atlantic salmon.

4. The physiological impacts of glochiodosis on brown trout change over the course of infection but appear to be generally mild. Splenomegaly (enlargement of the spleen) was observed only at circa one month post-exposure, and was positively correlated to glochidia abundance, though this effect was only transitory. Body size was positively correlated with glochidia abundance, but again only during the initial stages

of encystment. Hematocrit values, cryptic colouration, and ventilation frequency of brown trout hosts were not affected by glochidia loads.

5. A significant positive relationship was found between glochidia abundance and latency to leave shelter in brown trout, regardless of the number of days post exposure or host body size. This suggests that the parasitic stages of the freshwater pearl mussel can perhaps make the salmonid host more risk-averse, and therefore less likely to die through predation. This would benefit the non-trophically transmitted glochidia.

# Appendix

# Appendix

A description of histological techniques employed in this thesis, adapted from Humason (1979) and Lillie (1965).

## Sample Preparation

**Fixation**
Place the tissue in an excess of Freshwater Bouin's fixative. If there is a lot of blood or similar fluids in the sample, the fixative may change colour. Fixative can be changed (or "freshened up") if the discolouration is severe.

Allow at least 24 hrs. in the fixative.

**Embedding in Paraffin wax**
The tissue processor used in this study was a Shandon-Elliot Duplex Processer. All tissue processors vary in design, but essentially consist of a mechanism by which samples are moved from baths of various solutions after a set period of time. Note the melting point of Paraffin wax is 60°C. To embed the tissues (fish gills) used in this study we employed the following method:

1. 70% ethanol 1 hr.
2. 80% ethanol 1 hr.
3. 90% ethanol 1 hr.
4. 100% ethanol 1 hr.
5. Histoclear™ 1 hr.
6. Histoclear™ 1 hr.
7. Paraffin wax 2 hr.
8. Paraffin wax 2 hr.
9. Paraffin wax 2 hr.

Once the tissues have been infiltrated with Paraffin wax they are mounted in preparation for sectioning using a microtome. Section thickness will vary by tissue type and the requirements of the study. Sections are mounted on glass slides using a small drop of glycerin albumen solution and allowed to dry overnight.

# Staining Wax Sections with Cole's Haematoxylin and Alcoholic Eosin

1. Dewaxing HistoClear™ to remove any remaining traces of wax – 5-10 minutes
2. 100% alcohol – 2 minutes
3. 90% alcohol – 1 minute
4. Lithium Carbonate in 70% alcohol for 2-3 minutes
5. 70% alcohol for 1 minute
6. Cole's haematoxylin for 10 – 15 minutes
7. Wash off stain with tap water, then place slide in Scott's solution for 2 minutes. (Sodium bicarbonate and magnesium sulphate in order to stain nuclei blue.
8. Gently rinse slide with tap water and examine at low power (x10 magnification) with condenser diaphragm open; pat dry or wipe the underside of the slide to prevent it sticking to the stage. Do not allow slide to dry.
   - if overstained: differentiate in acid alcohol for 2-5 seconds
   - if understained: replace in Cole's and Scott's
9. 70% alcohol for 1 minute
10. 0.05% Alcoholic Eosin stain for 3-6 minutes.
11. Dip in 70% alcohol until stain ceases to wash out (a few seconds)
12. Examine under low power:
    - if overstained: differentiate in 70% alcohol
    - if understained: replace in eosin
13. Rinse with 90% alcohol for 10-15 seconds
14. 100% alcohol for 5 minutes
15. Clear in HistoClear™ for 5 minutes.
16. Mount with DPX (one small drop).
17. Allow to dry overnight.

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