



## Rearing of unionoid mussels

(with special emphasis on the Freshwater Pearl Mussel *Margaritifera margaritifera*)

Frank Thielen (editor)



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1. *Margaritifera margaritifera* in the river Our (Luxembourg). Photo: Fondation Hëllef fir d'Natur.
2. Juvenile *Margaritifera margaritifera*. Photo: Fondation Hëllef fir d'Natur.

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## Preface

The conservation of freshwater mussels is a major challenge as they belong to the most imperiled freshwater organisms worldwide. For instance from the 297 species recognized in North America, 213 are endangered, threatened, or of special concern. Freshwater mussels have an important ecological value as they improve water quality and provide nutrient and energy cycling in streams and lakes by filtering algae, bacteria, and organic matter from the water column. Extensive anthropogenic habitat alternations have led to dramatic population declines in freshwater mussels worldwide. Habitat degradation and water pollution not only harms mussel populations directly, but as the reproductive cycle of most naiads; mussels involve a fish species acting as an intermediate host, any shift or decline within the fish population has a negative indirect effect on the mussel population also.

As in North America, most European freshwater mussel species are highly threatened. The worst affected species are the freshwater pearl mussel (*Margaritifera margaritifera* L.) and the river mussel (*Unio crassus* L.). They both show a dramatic decline throughout their European distribution range. Both species are listed in Annex II of the Habitats Directive and *M. margaritifera* is also listed in Annex V (Directive 92/43/EEC). According to EU legislation (Directive 92/73/EEC; Directive 97/62/EEC) member states are obligated to protect and maintain the local populations of both species. Although many conservation programmes have been initiated all over Europe in the past, some mussel populations are nearing extinction.

One possibility to save the genetic diversity of these autochthonous populations could be to artificially breed juvenile mussels and introduce them to their native populations in order to stabilise them. Rearing the juvenile stages of *M. margaritifera* under controlled conditions could help compensate for an approximate 100% loss during the initial few years.

Many attempts and methods have been used to rear Freshwater Pearl Mussels and other mussel species under laboratory or semi-natural conditions across Europe during the last decade. To discuss the progress in the field of mussel propagation, an international seminar was organised within the LIFE Nature Project "Restauration des

populations de moules perlières en Ardennes – LIFE 05 NAT /L/000116" during spring 2008. LIFE NATURE is the EU's financial instrument for supporting nature conservation projects throughout the EU.

Many of the papers discussed during the seminar appear in this special issue of Ferrantia and include:

Articles describing the dramatic decline of *Margaritifera margaritifera* in Germany.

Ecological aspects of how to develop successful conservation strategies for the fresh water pearl mussel are presented.

Semi-natural and laboratory culture and propagation methods for the freshwater pearl mussel and other species including growth factors are discussed.

Questions regarding the release of captive bred animals are addressed.



# The freshwater pearl mussel (*Margaritifera margaritifera*) in Germany

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**Keywords:** Freshwater Pearl Mussel, *Margaritifera margaritifera*, Population decline, Ecological factors

## Abstract

The historical development of Freshwater Pearl Mussel research is shortly shown and the dramatic decline of many population during the last decades is described. Biological and ecological surveys in German Pearl Mussel rivers showed that only few populations survived. These

population are however overaged and have come close to extinction. The reason for the Pearl Mussel decline are discussed and an interpretation of the factors leading to the dramatic decline is done. Suggestions on how Pearl Mussel rivers could be improved are given.

## Résumé

Le développement historique de la moule perlière des eaux douces est brièvement montrée et le déclin dramatique pendant les dernières décades est décrite pour plusieurs populations. Le suivi biologique et écologique de cours d'eau allemands contenant des moules perlières montre que seulement peu de populations ont

survécues. Ces populations avec de nombreuses moules très âgées sont proches de leur extinction. Les raisons du déclin des populations sont discutées et l'interprétation des facteurs responsables de leur déclin sont détaillées. Des suggestions pour améliorer les cours d'eau des moules perlières sont données.

## Zusammenfassung

Ein kurzer Abriss über die historische Entwicklung der Flussperlmuschelforschung wird aufgezeigt sowie ein Überblick über den dramatischen Rückgang der Bestände innerhalb der letzten Jahrzehnte. Biologische und ökologische Untersuchungen in deutschen Perlbächen haben gezeigt, dass nur sehr wenige Populationen bis zum heutigen Zeitpunkt überlebt haben. Viele

dieser Populationen sind zudem überaltert und stehen kurz vor dem Aussterben. Die Gründe für den starken Rückgang der Flussperlmuschel werden diskutiert und eine Interpretation der Faktoren, welche den starken Rückgang hervorgerufen haben, wird durchgeführt. Ratschläge, wie der Zustand in Perlbächen verbessert werden könnte, werden gegeben.

## Introduction

Interest in the freshwater pearl mussel (*Margaritifera margaritifera*) has a long historical background (Pfeiffer 1914). For many centuries that interest relied mainly on the capability of the species to produce genuine natural pearls. Early studies mainly focussed on the production of the shell and the pearls as well as on the distribution and occurrence of the species.

Investigations into the anatomy and biology, above all the reproduction mode of the species, only began with the rise of modern science (for a review see for instance Harms 1908). Antonie van Leeuwenhoek (24 October 1632-26 August 1723), a Dutch tradesman and scientist, who used microscopes crafted by himself, was the first to observe small, mussel-like organisms on the gills of various species of mussels of the genera *Unio* and *Anodonta*. He believed those organisms to be juveniles and inferred the host mussels to be viviparous. More than 100 years later, Martin Heinrich Rathke (25 August 1773-3 September 1860), an anatomist working in Dorpat and Königsberg, reported on the presence of tiny parasites on fishes, which parasites he described as *Glochidium parasiticum* (in fact Unionoidea larvae), but without recognizing the relationship between those parasitic larvae and the mussels. The zoologist Carl Gustav Carus (1789-1869) (1832) described the development of the glochidia (larvae) within the eggs attached to the gills of the adult female mussels. The fact that the glochidia must fulfill their embryonic development on the gills (freshwater pearl mussel) or the fins (Unionidae) of various host fishes was only recognized much later.

It is noteworthy that in the course of history many governmental ministries felt that they should take care of the freshwater pearl mussel, but their interest focussed almost exclusively on the reserved right to collect the mussels and the pearls they produced.

Following the decline of the pearl mussel populations, a decline which was repeatedly reported as soon as in the early 19<sup>th</sup> century (see for instance Baer 1995), the pearl fishery decreased progressively and finally came to an end. Late official pearl mussel fishery activities in Germany were carried out in the 1950s in Bavaria (as testified by the announcements of public auctions in the govern-

mental Bavarian journal) and the mid-1960s in the Odenwald (documented in a film).

Finally, the interest in the pearl mussel and its conservation vanished and the existence of the species almost fell into oblivion.

Intensive investigations into the occurrence of the pearl mussel and its habitats were carried out after 1945 (Hertel 1959; Baer 1995). During the late 1960s, following the pioneering work of Dr med. Wolff-Dietrich Bischoff (†, Hannover) in the Lüneburger Heide, conservation activities and studies resulting in a better knowledge of the life-cycle and ecology of the species were carried out. Later studies reported on the causes of the decline of the populations (e.g. Utermark 1973; Jungbluth & Lehmann 1976, Jungbluth & Utermark 1981). The first conservation activities were carried out in a number of regions (the first one by W.-D. Bischoff, W. Utermark und K. Wächtler in the Lüneburger Heide). More recently, from 1985 to 1987, a survey with the title "Ökologische Standortüberprüfung<sup>®</sup>" was carried out throughout Germany by the "Projektgruppe Molluskenkartierung<sup>®</sup> (Neckarsteinach, Schlierbach since 1994)". Jungbluth (1988) reported on the results of that survey in the Rhine region. A number of other naturalists continued this research after 1988, so for instance the research groups led by K. Wächtler (Hannover) and G. Bauer (Bayreuth, later Freiburg) or they continued working on the pearl mussel project in other regions in Germany.

## Material and methods

### Distribution of the pearl mussel in Germany

The distribution of the pearl mussel in Germany, and in Central Europe in general, was already well known. Similarly, the general decline of the populations at the majority of the localities since the early 19<sup>th</sup> century had been thoroughly investigated (see for instance Hertel 1959, for the Saxonian Vogtland; that work was continued by Baer (1995)).



The initiatives of Dr med. W.-D. Bischoff in the Lüneburger Heide and Prof. Dr H. Grohs in the environs of Linz/Austria revived the research on the pearl mussel in central Europe. Several groups of researchers started investigations in a number of regions, so for instance in the region of the Vogelsberg (Jungbluth & Lehmann 1976; Utermark & Jungbluth 1981).

Conservation measures were implemented in the Lüneburger Heide and similar projects were carried out the German Mittelgebirge (low mountain ranges). From 1985 to 1987 the "Projektgruppe Molluskenkartierung<sup>®</sup> (Neckarsteinach)" carried out the first investigation on the distribution of the species across the Federal Republic of Germany, this through a newly developed method called "Ökologische Standortüberprüfung<sup>®</sup>." The investigators checked all extant populations, the remains of populations as well as all the localities recorded in both the literature and the scientific collections (the so-called analysis of the historical occurrence in Germany). Negative results were also reported (photographic documentation). All in all, around 300 sites were visited. As a result, in 93 of the 269 streams that were formerly known to harbour pearl mussels, only ruins of former populations or isolated individuals were found (e.g., Jungbluth 1988).

## Results

### Ecological results

The literature contains only rare data on the environmental conditions under which the species was found living. In general, only the water temperature and hardness were recorded. This makes that the historical conditions under which the species lived are poorly known. The available data characterized the pearl mussel as highly oligotitanophilic (i.e. linked to waters with a very low carbonate content). After 1945, extensive investigations into the limnology of streams, those harbouring pearl mussel populations included, were carried out. In the course of the above mentioned "Ökologische Standortüberprüfung-survey", analyses of the biological water quality were carried out at the localities (n=93)

where living pearl mussels had been found. As a result, a rich quantity of data was collected, thus characterizing the present-day conditions in which the pearl mussels live. However, it should be noticed that these data were collected from streams harbouring ruins of pearl mussel populations and that they do not describe at all the ecological conditions of the streams in which the striving historical pearl mussels lived.

Table 1 is based on the data available from the above mentioned analyses. It presents the ecological conditions linked to the presence of the pearl mussel in streams of eastern Bavaria and the Mittelgebirgs-transect.

### Biological results

The biological results, as presented by Jungbluth (1996), can be summarized as follows.

All the populations recorded were over-aged and thus lacked the minimum of juvenile individuals needed to maintain or rebuild stable populations. The 1985-1987 survey showed that the majority of populations consisted of individuals aged 40 and more years. Today, 20 years after that survey, the situation has remained unchanged. The adult individuals of the still extant populations continue to reproduce, but the proportion of juvenile animals has remained unchanged. The adult stock has shifted towards the 60+ years category. The stock of young adults is small and will not allow the population to remain stable in numbers over the coming years.

The rearing of the larvae in the laboratory and also the infection of host fishes with the glochidia (as for instance by the method developed by G. Wellmann) has been successfully carried out. However, once detached from the host fish, the development of the young mussels within the hyporheic interstitial, still leads to high losses in numbers, the causes for this loss remain to be studied.

**Table 1:** Ecological demands of the Freshwater Pearl Mussel in eastern Bavaria and the Mittelgebirge-transect: biotic and abiotic factors.

| <b>Abiotic factors</b>                 |                                                                               |
|----------------------------------------|-------------------------------------------------------------------------------|
| <b>I. Watercourse</b>                  |                                                                               |
| Location                               | Fichtelgebirge, Bayer. Wald / Mittelgebirge-transect                          |
| Geological bedrock                     | Granite, Gneiss,                                                              |
| Biotic zone                            | Submountain                                                                   |
| Altitude                               | up to 500 m                                                                   |
| Decline                                | 0,2 - 0,7 %                                                                   |
| Discharge                              | Pluvial                                                                       |
| River type                             | Low Mountain River/ upper and lower salmonid region                           |
| <b>II. Ecomorphology</b>               |                                                                               |
| Petrography                            | Granite, Gneiss                                                               |
| Alignment                              | Meander and straight stretches                                                |
| Lining                                 | without                                                                       |
| Macrooptical pollution                 | Domestic waste and building rubble                                            |
| Flow conditions                        | low - normal                                                                  |
| River width                            | 1 - 5 m                                                                       |
| Flow pattern                           | Calm - turbulent                                                              |
| Average current                        | 20 - 40 cm/s                                                                  |
| River bottom current                   | - Juveniles [< 20 Years]: 0 - 5 cm/s<br>- Adults [> 50 Years]: 2 - 9 cm/s     |
| Flow                                   | > 20 l/ sec                                                                   |
| Water depth                            | > 10 cm                                                                       |
| Visibility depth                       | 0,1 - 0,3 m                                                                   |
| Turbidity                              | low                                                                           |
| Choriotope                             | Psammal - Mikrolithal                                                         |
| Shading                                | 25 - 75 % [and more]                                                          |
| Bank structure                         | Riparian vegetation, shading 60 - 100 %<br>Structured- well structured        |
| Phythal [proportion]                   | < 6 - 50 %                                                                    |
| Pelal                                  | < 6 - 50 %                                                                    |
| Psammal                                | > 6 - 12 %                                                                    |
| Lithal                                 | 12 - >50 %<br>10 - 30 cm                                                      |
| Riparian vegetation                    | Autochthonous species on both river banks (Wood or extensive used green land) |
| Agricultural use of the catchment area | < 30 %                                                                        |
| <b>III. Water Chemistry</b>            |                                                                               |
| Water temperature                      | 0 - 23 °C                                                                     |
| pH                                     | 6,7 - 8,8                                                                     |
| O <sub>2</sub>                         | 7,6 - 16,2 mg/l                                                               |
| Conductivity                           | < 37 - 194 µS *cm <sup>-1</sup> [20 °C]                                       |
| Calcium                                | < 8 - 10 mg/l                                                                 |
| Total-phosphate                        | < 20 - 35 P µg/l                                                              |

|                               |                                                                                                                                                |
|-------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------|
| KMnO <sub>4</sub> -demand     | < 10 mg/ l                                                                                                                                     |
| BSB <sub>2</sub>              | 0,88 - 4,05 mg/ l O <sub>2</sub>                                                                                                               |
| BSB <sub>5</sub>              | < 1,5 mg/ l O <sub>2</sub>                                                                                                                     |
| Ammonium/ NH <sub>4</sub>     | < 0,000 - 0,077 [0,1] mg/ l                                                                                                                    |
| Nitrate/ NO <sub>3</sub>      | < 2,79 - 21,60 mg/ l                                                                                                                           |
| Nitrite/ NO <sub>2</sub>      | < 0,003 - 0,008 mg/ l                                                                                                                          |
| Chloride/ Cl                  | < 0,5 - 19,2 mg/ l                                                                                                                             |
| Acid capacity Ks 4,3 m mol/ l | 0,2 [0,1]                                                                                                                                      |
| Total hardness                | < 2,6 - 5,0 mval/ l                                                                                                                            |
| Carbon hardness               | < 0,9 - 4,0 mval/ l                                                                                                                            |
| DOC                           | 1,8 - 3,3                                                                                                                                      |
| <b>Biotic factors</b>         |                                                                                                                                                |
| Saprobic index                | < 1,7                                                                                                                                          |
| Fauna                         | Macrozoobenthos of low mountain rivers                                                                                                         |
| Water quality class           | I - II (II)                                                                                                                                    |
| Host fish                     | autochthonous brooktrout ( <i>Salmo trutta fario</i> ) with natural population distribution and reproduction. High abundance of 0+ and 1+ fish |

## Discussion

### Declining populations - an attempt of interpretation

The dramatic decline of the pearl mussel populations may be due to the causes described below. The attempt of an interpretation of the decline is derived from the present author's 40 years period of experience with the Unioidea in general, and it clearly also applies to the decrease of the thick shelled river mussel (*Unio crassus*) populations.

#### Stage I: Agricultural intensification

After 1945 the immigration of large numbers of refugees from the eastern Europe and the decline of farming due to the Second World War led to a dramatic shortage of food. In reaction, agriculture in Germany was rebuilt and extraordinarily intensified. The renewed farming methods included the heavy usage of both fertilizers and pesticides, DDT included. As a consequence, noxious substances were incorporated into the sediments of rivers.

#### Stage II: Water supply and industrial activity

Fertilizers were used in parallel to heavy hydraulic engineering projects within the open landscapes, this in order to expand farm fields, a process which included the drainage of wetlands used for the extensive summer pasture in the Mittelgebirge. Also, the beds of rivers and even streamlets were straightened, in many places the stream water was evacuated into the underground, this in order to free the farming land rapidly from excessive water and to use the streams for the evacuation of sewage waters. Additionally, these activities guaranteed the water supply for the industry.

The effects of hydraulic engineering were exclusively evaluated from the agricultural and industrial points of view. In general, water quality and the natural structure of the landscapes were not taken into consideration, and both clearly suffered from those engineering activities. As a consequence, the usage of fertilizers, through eutrophication and massive algal blooms, locally and regionally caused problems to the water purification.

### Stage III: The ecological point of view

As a consequence of the ecological deterioration described above, surveys of the water quality of natural water bodies, mainly of rivers, were initiated on a regular basis (for instance on a five years base). Also, sewage treatment was initiated, thus markedly improving the water quality of streams. Sewage treatment presently continues to be improved through the addition of further stages to the treatment.

However, in parallel to the disappearance of heavily polluted stretches of streams, there was a loss of formerly unpolluted, i.e. oligotrophic stretches of rivers. In recent years, a number of German States have introduced an exhaustive survey of the quality of natural water bodies. These surveys aim at determining the ecological quality of the rivers and to make possible the subsequent improvement of that quality. This means that the original structure of rivers has to be re-established, the former hydraulic engineering having caused repeated floods in the regions of the lower course of rivers.

The conclusions derived from the records extending over the recent 40 years period of the Unionoidea in the majority of the German States are as follows.

In the stage I mentioned above (agricultural intensification), fertilizers, pesticides, DDT included, and other chemicals were heavily used without consideration of the ecological consequences. The excessive usage of fertilizers, stimulated by the shortage of food, which shortage was due to the massive human immigration from eastern Europe into West Germany is well documented.

A well known example of this consequences was the dramatic decline of the populations of the Peregrine Falcon (*Falco peregrinus*), down to one single pair of mates in the valley of the Neckar in Hesse. During many years this couple of birds was incapable of producing offspring. It was not until the reduction of the quantity of fertilizers and other heavy environmental pollutants had been obtained, that viable and even expanding populations of the Peregrine Falcon existed again in central Germany, this also thanks to the extraordinary commitment of many, mainly benevolent, bird conservationists.

The decline of the pearl mussel populations obviously results from the same ecological changes. From the fact the first stages of the life-cycle of the species are untouched (this could be shown at a number of localities), it must be concluded that the modified riverine substrate is the cause of the reduced number of young mussels. However, the pearl mussel problem appears to be more complex than expected from the conditions described above.

#### I. The present status of the pearl mussel rivers: the burden of the past

Various attempts to analyse the structure and quality of the sediments, so for instance in the Our river in Luxembourg, were so far unsuccessful, this because of the fact that various issues of the methodology could not be addressed properly.

Until the precise ecological conditions within the substrate will be clarified, we must provisionally admit that a number of pollutants, among them fertilizers as well as pesticides, have accumulated therein over the last several decades, and that these pollutants continue to be released into the flowing water. Harmful modifications of the grain size of the deposits are also possible. More research into the quality of the substrate and the adjacent hyporheic interstitial habitat are needed to find out the original ecological conditions.

In the first place the regeneration of the original conditions of the riverine substrate should be obtained. If this cannot be achieved, all other conservation measures in favour of the pearl mussel, and similarly also the hard shelled mussel, are most probably doomed to failure.

It is possible that within the next future the pollutants will progressively and completely be eliminated from the substrate. However, it remains to be seen if then a sufficient number of reproductively active individuals of the pearl mussel will still be present to ensure the recolonisation of the formerly occupied stretches of rivers.

#### II. The present status of the pearl mussel rivers: Trophic status

Recent investigations carried out by Hruška (1995), Hruška & Bauer (1995) and Schreckenbach (1995) have attracted the researchers' attention towards 'new' aspects of the habitat and the feeding mode of the mussels.

- The new questions, among others, are: What were the consequences of the hydraulic engineering on the load of suspended particles in the water? Were these changes positive or negative for the development of the pearl mussels?
- And: what were the ecological consequences, in terms of food availability and composition, of the hydraulic deepening of the river beds for the juvenile and the adult pearl mussels, respectively?

Site specific answers have to be found for these questions.

Could the success story of the Peregrine Falcon be an example for the conservation of the pearl mussel populations and their recolonisation of abandoned sites?

The above report on the extant ruins of populations of the pearl mussel shows that the reduced survival of the young mussels within the riverine substrate is the most sensitive stage in the life of the pearl mussels, and that this knowledge is also the key to successful conservation measures.

On the one hand, the basal knowledge allowing the conservation of the species is still far from complete. On the other hand, the ongoing decline of the populations requires the establishment of an emergency program.

In recent years and at a number of places, brown trout (*Trutta salmo fario*) are infected, under semi-natural conditions and following the method developed by Gustav Wellmann, with larvae of the pearl mussel. This method is practiced for more than 40 years in the Lüneburger Heide and is also applied in Bavaria and on the river Our in Luxembourg. There exist however unresolved issues about the semi-natural infections regarding the structure of the rivers where this method is applied:

- What is the structure and composition of the sediments of the rivers into which the young mussels are released?
- Why is the abundance of young mussels, if they exist at all, so low?
- What is presently the trophic situation of the young mussels within the substrate and the immediately following stage of life?

## Outlook

To meet the expected success, conservation projects for the Unionidea and the pearl mussel in particular, must be planned and executed over the long-term. Indeed, one should be aware that sexual maturity in the pearl mussel is only reached at the age of 20 years or even beyond. Special attention should be paid to the conservation and management of the habitats.

A common conservation project should be implemented throughout the concerned German States - this was so far not possible because of the federal political system in Germany, in which system nature and species conservation are in the hands of the States rather than the federal government - in order to enhance collaboration and to distribute the tasks to be done among the scientific teams. This would make the realization of conservation projects possible at various localities extending over a relatively large number of years, projects that would not be linked to the duration of parliamentary sessions.

Pioneering work in favour of the pearl mussel was done by W.-D. Bischoff, W. Utermark und K. Wächtler in Lower Saxonia. The State of Bavaria, because of its large area, the number of pearl mussel rivers and abundance of the species there, has a central role to play for the conservation of the species in Germany. It is suggested here, that Bavaria should take over the role of a coordinator of the conservation projects in Germany in general.

The package of conservation measures taken in Luxembourg, and also the project realized in North Rhine-Westphalia, are particularly noteworthy. Both projects should be incorporated into a common and large project. Since the German reunification, the Free State of Saxony has initiated a species conservation project based on both national and international cooperation. The Projektgruppe Molluskenkartierung<sup>®</sup> is ready and willing to assume the role of central coordination and also a tutorial function. Both activities are based on the theoretical and practical knowledge acquired throughout the last several decades. The project group offers its help and willing to cooperate with other groups.

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# Application of a five-stage field key for the larval development of the freshwater pearl mussel (*Margaritifera margaritifera* Linné, 1758) under different temperature conditions - A tool for the approximation of the optimum time for host fish infection in captive breeding

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## Abstract

As the freshwater pearl mussel (*Margaritifera margaritifera* Linné, 1758) is highly endangered throughout its distribution area, conservation programmes are carried out in many countries, dealing mostly with semi-natural captive mussel breeding. The crucial stage of such breeding projects is the fixing of the ideal time for collecting mature mussel larvae for the infection of the provided host fish. The present study introduces a five-stage field key for the determination of clearly discernible developmental stages of mussel larvae. By means of this key, the developmental progress at any certain instant of time can be determined, and the remaining time till the release of mature larvae can be estimated.

As larval development in poikilothermic animals always depends on the temperature of the surrounding medium, three case studies at markedly different thermic situations are provided for Central European conditions. An average, a comparably cool and a relatively warm summer have been compared with regard to larval development.

In the extraordinarily warm summer of 2007, a succession of two complete reproduction cycles of the normally strictly univoltine freshwater pearl mussel was detected. Reasons and possible effects are discussed.

## Résumé

La moule perlière (*Margaritifera margaritifera* Linné, 1758) est au bord de l'extinction bien que protégée. Il y a beaucoup de projets dans tout l'Europe qui essaient à sauver les populations restantes en infectant des truites farios avec des larves du mollusque, qui se développent à l'abri des branchies de ces poissons. Le problème essentiel, c'est trouver le moment exact pour l'infection. Celle recherche-ci présente une clé d'identification pour

les stades de développement avec laquelle cinq stades peuvent être déterminés directement sur le terrain. Le progrès du développement peut être identifié à chaque instant quelconque par le biais de la clé pour estimer le temps restant jusqu'à la libération des larves mûres.

Comme le développement des animaux poikilothermes relève de la température de l'eau, trois études modèles

sont mises à disposition. Elles traitent trois situations thermiques qui se distinguent essentiellement l'un de l'autre et qui sont typiques d'Europe centrale. Il s'agit d'une année moyenne, une année relativement chaude et une année comparativement froide.

### Zusammenfassung

Da die Flussperlmuschel (*Margaritifera margaritifera* Linné, 1758) in ihrem gesamten Verbreitungsgebiet als höchst gefährdet zu betrachten ist, werden in vielen Ländern Artenschutzprogramme durchgeführt, die vor allem auf die halbnatürliche Nachzucht von Jungmuscheln im Labor ausgerichtet sind. Die sensibelste Phase in diesen Nachzuchtprojekten ist die Festlegung des richtigen Zeitpunktes für die Infektion von Wirtsfischen mit den parasitären Larven der Flussperlmuschel.

In der vorliegenden Arbeit wird ein fünfstufiger Bestimmungsschlüssel für klar gegeneinander abgrenzbare Entwicklungsstadien von Flussperlmuschelglochidien vorgestellt. Mit Hilfe dieses im Freiland anwendbaren Schlüssels kann zu jedem beliebigen Zeitpunkt der Entwicklungsfortschritt der Muschellarven festgestellt

En été 2007 les températures extraordinairement hautes ont conduit à deux cycles de reproduction particuliers, quoique normalement, les moules perlières soient univoltins. Probables raisons et effets possibles sont discutés.

und die Zeit bis zum Erreichen der Infektionsreife abgeschätzt werden.

Da die Geschwindigkeit der Larvalentwicklung bei allen wechselwarmen Tieren stark von der Umgebungstemperatur abhängt, werden drei für zentraleuropäische Verhältnisse typische Temperaturszenarien miteinander verglichen. Ein durchschnittlicher, ein vergleichsweise kalter und ein auffällig warmer Sommer wurden in Hinblick auf die Larvalentwicklung in einer natürlichen Flussperlmuschelpopulation miteinander verglichen.

Im außergewöhnlich warmen Sommer 2007 wurden zwei separate, aufeinanderfolgende Fortpflanzungszyklen bei der sonst streng univoltinen Flussperlmuschel beobachtet. Mögliche Gründe für dieses Phänomen und daraus resultierende Effekte werden diskutiert.

## Introduction

The freshwater pearl mussel (*Margaritifera margaritifera* Linné, 1758) is one of the most threatened species in the Northern hemisphere (Young et al. 2001), especially in Europe. Once having occurred in lime-poor running waters all over Northern, Central and Western Europe in vast numbers, its populations have declined for several decades and still keep decreasing rapidly. Disillusioning reports of dropping mussel numbers arrive from virtually every country of its distribution area, including amongst others England and Wales (Chesney & Oliver 1998), Northern Ireland (Beasley & Roberts 1996), Scotland (Cosgrove et al. 2000), Ireland (Moorkens 1999), Germany (Vandré et al. 2000), the Czech Republic (Hruška 1998), Spain (Bouza et al. 2007), Latvia (Rudzīte 2005), Belgium (Terren et al. 2006), Luxemburg (Jungbluth 1988) and Austria (Scheder & Gumpinger 2007).

The reasons for the grave situation are manifold. Firstly, the freshwater pearl mussel shows a very complicated and peculiar reproduction mode. The female mussel produces several million of parasitic larvae, so-called glochidia, which are borne within special formations of the parental gills (Young &

Williams 1984). After having completed their development inside their mothers' shells, the larvae are expelled into the surrounding water and are immediately inhaled by brown trout (*Salmo trutta* Linné, 1758), the preferred host fish species for Central European freshwater pearl mussel populations (Wächtler et al. 2001). Once attached to the host's gills, they cling to the respiratory tissue that soon starts overgrowing the parasite. The glochidia overwinter inside the cysts, undergo metamorphosis in late spring, then disengage from their hosts and drop to the riverbed. For at least five years, they live inside the hyporheic interstitial (Bischoff et al. 1986), before they join their adult conspecifics on the surface of the riverbed. It is obvious that such a meticulous reproduction cycle is highly vulnerable. The crucial stage in the life cycle is the period during which the juvenile mussels live in the interstitial (Geist 1999). Geist & Auerswald (2007) identified the characteristics of the stream substratum, the depth profile of the redox potential, the penetration resistance of the stream bottom and the physical connectivity of free-flowing water and the interstitial zone as substantial factors for successful pearl mussel recruitment. Due to the intensive agricultural use of the catchment areas (combined with over-



fertilization and the clear-cut of alluvial forests), enormous loads of fine sediments are transported into the river systems, causing heavy siltation effects in the interstitial (Altmüller & Dettmer 1996). The pores in which the juvenile mussels dwell are clogged, and the supply of nutrients and oxygen is cut off. The young mussels hence either suffocate or starve to death, and the populations concerned show a remarkable excess of age.

In all the countries mentioned above, protection projects are currently being carried out in order to prevent the mussel from becoming extinct. As the present study bases on surveys carried out in Austria, the specific situation of the Austrian freshwater pearl mussel populations is depicted below.

In Austria, the distribution area of the freshwater pearl mussel has always been restricted to the northern parts of Upper and Lower Austria, where it used to occur in enormous densities (Gumpinger et al. 2002). Nowadays, only some isolated scattered beds are left, the largest ones not exceeding a few hundred mussels (Scheder & Gumpinger 2008). Most populations lack juveniles, as the natural reproduction has not resulted in a sufficient amount of viable juvenile mussels throughout the past decades – mostly due to the severe siltation of the river beds. In order to conserve the few remaining populations, a large-scale protection programme has been carried out in the Upper Austrian River Waldaist for over ten years, dealing mainly with the support of the natural reproduction by semi-natural breeding. This very population was chosen, as Moog et al. (1993) have described the River Waldaist as "the best remaining freshwater pearl mussel stream in Austria with a population of highest relevance and worthiness of protection". Geist & Kuehn (2005) examined the population's genetic aspects and proved a closer relationship to the population

in the River Kamp in Lower Austria than to any Upper Austrian population. They therefore suggest regarding the populations of the River Waldaist and the River Kamp as a separate conservation unit within the Danube drainage.

In the course of the species protection project, a method for approximating the optimum time for the artificial infection of the host fish has been developed by describing five developmental stages that are easily discriminable in the field.

## Material and Methods

Investigations were carried out in the largest remaining Upper Austrian mussel population, which is located in the River Waldaist. Physicochemical characteristics of the river are listed in Tab. 1 (data provided by the Office of the State Government of Upper Austria, Department of Surface Water Management (Linz)).

Between 2005 and 2007, the gestation rate of adult mussels from the River Waldaist and the degree of development of their larvae were monitored every summer. The survey aimed at gaining mature larvae for the semi-natural infection of cultured fish that were to be released into appropriate river systems immediately after infection. In order not to miss the larval release, examinations were carried out at periods constantly decreasing in length. At the commencement of each reproduction cycle, controls were performed once a week. With preceding development, intervals were shortened after each monitoring, until, during the final stage of the observations, daily samples were taken.

Several mussels were taken out of the substrate cautiously, and slightly opened by means of special pliers. If gravidity could be attested – in terms

**Tab. 1:** Physicochemical characteristics of the River Waldaist (average over 33 months: November 2006 - July 2009).

|                                           |                                            |                                    |                                    |                                  |                                  |                                  |                                   |
|-------------------------------------------|--------------------------------------------|------------------------------------|------------------------------------|----------------------------------|----------------------------------|----------------------------------|-----------------------------------|
| MQ<br>(m <sup>3</sup> s <sup>-1</sup> )   | Conductivity<br>(µS cm <sup>-1</sup> )     | pH                                 | Magnesium<br>(mg l <sup>-1</sup> ) | Calcium<br>(mg l <sup>-1</sup> ) | Total<br>hardness                | DOC<br>(mg l <sup>-1</sup> )     | Oxygen<br>(mg l <sup>-1</sup> )   |
| 3,12                                      | 102                                        | 7,32                               | 1,64                               | 9,33                             | 1,69                             | 6,56                             | 11,30                             |
| Orthophos-<br>phate (mg l <sup>-1</sup> ) | Phosphorous<br>Total (mg l <sup>-1</sup> ) | Potassium<br>(mg l <sup>-1</sup> ) | Sodium<br>(mg l <sup>-1</sup> )    | Sulfate<br>(mg l <sup>-1</sup> ) | Nitrate<br>(mg l <sup>-1</sup> ) | Nitrite<br>(mg l <sup>-1</sup> ) | Ammonium<br>(mg l <sup>-1</sup> ) |
| 0,015                                     | 0,035                                      | 1,20                               | 6,68                               | 9,21                             | 1,13                             | 0,004                            | 0,44                              |

of the presence of a distinct, yellowish cell mass within the gill tissue – samples of larval material were taken with the aid of a disposable syringe. The samples were then observed employing a hand held optical microscope (Enhelion Micron pro, 160-fold magnification). Micrographs were taken by means of a digital camera (Canon PowerShot G6).

Conspicuous changes in larval morphology, morphodifferentiation and mobility over the course of time were registered. Clearly distinguishable developmental stages were characterized verbally and sketched from micrographs by means of a technical pen.

In order to find out about a correlation between the rate of larval development and the water temperature, a temperature measuring probe (Te.M.P. by blattfisch) was installed in the River Waldaist directly next to the mussel bed.

## Results

The development of the glochidia turned out to pass through five stages that are easy to discriminate morphologically in the field by means of basic light microscopy. The exact process of larval development in *M. margaritifera* has been described explicitly by Scharsack (1994) by use of electronic microscopy. But in the course of applied conservation projects, more simply discernible stages that can be distinguished by means of basic equipment can be regarded as a significant work simplification. The five "field stages" are described and pictured below.

**Stage 1.** The first distinguishable stage can be characterized as a spherical, compact mass of cells without any further differentiation (Fig. 1a). The larva is enclosed within a thin, transparent, globular-shaped egg shell. The first stage is totally immobile. Individuals are often closely attached to their neighbours, forming long strings or even tissues of larvae. At this early stage of development, the larva has already reached its final dimensions of about 40 – 70 µm in diameter. In the course of the following stages, only further differentiation, but no more growth takes place. Stage 1 is the most long-lasting of the five stages described in this study.

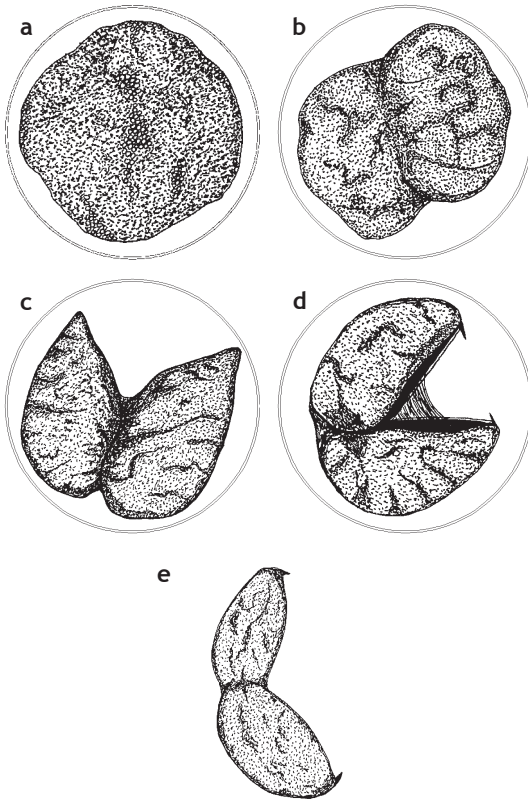
**Stage 2.** As soon as a larva has reached the second stage, distinct constrictions become clearly visible along the median axis of symmetry (Fig. 1b). Thus, the left- and right-hand side of the larva can be distinguished for the first time at this stage. The second stage is still enclosed within the egg shell and fully immobile.

**Stage 3.** In the third stage, the future mussel shells develop. The typical semi-spherical, hollow structures, in which the body is to be enclosed, are formed. Viewed ventrally or dorsally, the two valves appear drop-shaped (Fig. 1c), as the median and lateral edges of each valve converge and meet at the tip at an acute angle. The final shape of the glochidium is fixed at the end of the third stage. Still, no movement can be detected at this stage, and the larva still lies inside its transparent egg shell.

**Stage 4.** Hardly any major differentiation occurs between stage 3 and stage 4, but larvae that have reached the fourth stage start moving inside their egg shells. They perform snapping movements by actively opening and closing their shells. As development goes on, the snapping becomes more and more frequent. Membranous structures can be noticed between the valves, being stretched when the larva opens its shells (Fig. 1d). From the dorsal or ventral view, two tooth-like projections can be perceived at the margin of each valve.

**Stage 5.** Larvae hatch from their egg shells and start moving around freely (Fig. 1e), snapping heavily. The stout spines at the apical end of each shell are now clearly visible. When a strongly diluted solution of sodium chloride is added, the larva closes its shells and does not open them anymore. This reaction is explained by the fact that free fifth stage larvae have to find an appropriate host fish and attach to its gills as quickly and strongly as possible. As in fish a large part of the salt metabolism is performed via the gills (Smith, 1929), a high concentration of ions in the surrounding water indicates the presence of adequate host tissue to the glochidium.

Only stage 5 larvae are capable of infecting host fish successfully. In semi-natural breeding it is therefore inevitable to collect larval material exactly at the right time. If glochidia are gained too early, larvae cannot cling to the host's gills properly, as they are still encased in their egg shells. Waiting too long for mature larvae on the other hand may result in a total loss of glochidia, as gravid



**Fig. 1:** Developmental stages of the freshwater pearl mussel; a: stage 1 (age: 4 days); b: stage 2 (age: 16 days); c: stage 3 (age: 23 days); d: stage 4 (age: 28 days); e: stage 5 (age: 30 days). Age specification for mean water temperatures of 14.3 °C in August.

mussels of the same population expel their larvae highly synchronically. The observation of the larval development and the assignation to one of the five described stages can help to make a rough estimate of the time that is left until the release of mature, infectious larvae. As the developmental rate in poikilothermic animals always depends on the temperature of the surrounding medium, the prediction of the ideal time for infection must always regard the prevalent temperature regime of the watercourse. The varying time requirement for the completion of development under different temperature conditions is depicted below on the basis of three exemplary thermal situations for Central European conditions.

The years 2005, 2006 and 2007 represented three exceedingly different years regarding the respective water temperature regime (Tab. 2).

2005 can be referred to as an "average" year with air and water temperatures typical of the warm-moderate, Central European climate. The mean water temperature in August amounted to 14.3 °C, the mean annual water temperature to 8.1 °C. In such a year, the first occurrence of stage 1 larvae can be expected at the end of July or beginning of August (Fig. 2, top left). As the first stage is the most long-lasting one in the course of development, stage 1 larvae can be found for about two weeks. Under average temperature conditions, stage 2 glochidia will be present from the middle of August onwards. The rate of development is getting slightly faster with every stage; that is why stage 3 larvae are likely to replace stage 2 larvae within about eight to ten days. The further development proceeds even more quickly. The transition from stage 3 to stage 4 takes place within half a week, so does the final step from stage 4 to stage 5.

The summer of 2006 was, by contrast, remarkably cold. The mean water temperature in August amounted to only 13.0 °C. Though first stage larvae could be detected at the beginning of August (just like in the average summer of 2005), the development of stage 2 larvae was markedly retarded (Fig. 2, top right). They could not be proven until the third week of August, their development thus

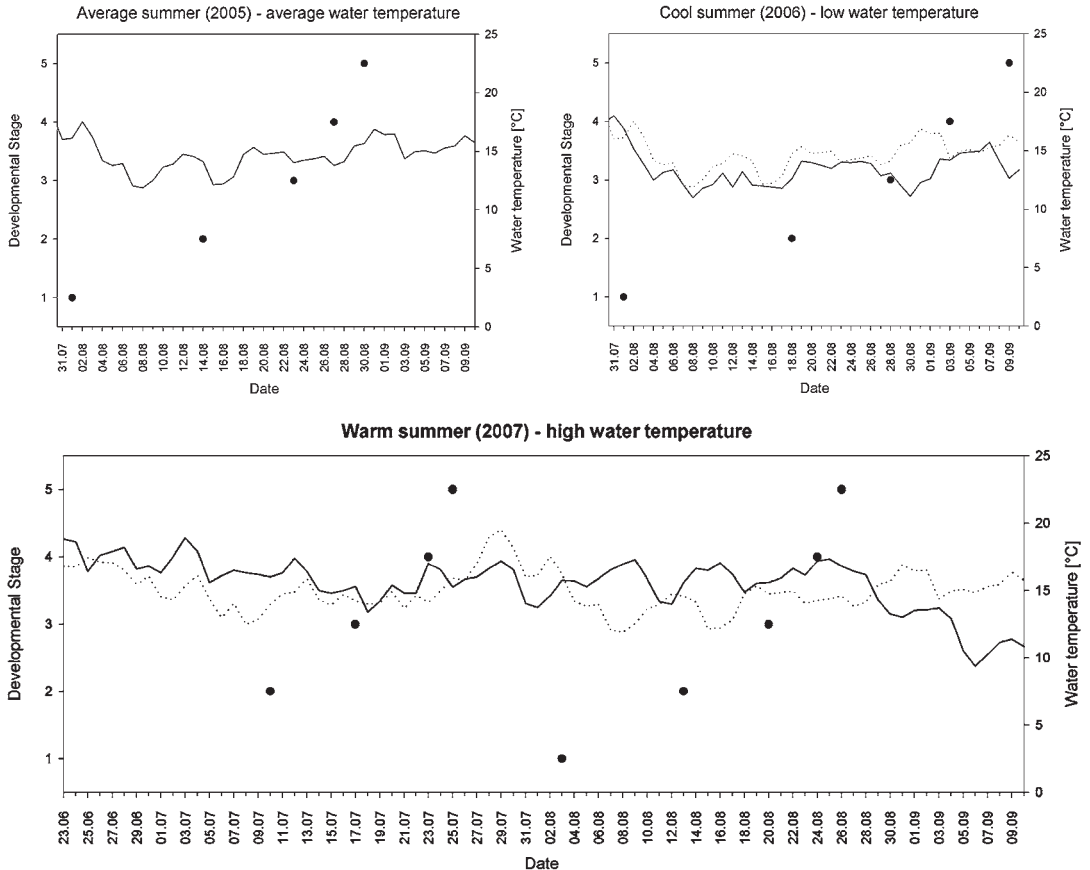
**Tab. 2:** Temperature characteristics of the three different temperature scenarios (2005, 2006, 2007).

|                                       | Mean water temperature in August (°C) | Number of days with water temperature: |               |               |               |        |
|---------------------------------------|---------------------------------------|----------------------------------------|---------------|---------------|---------------|--------|
|                                       |                                       | <14 °C                                 | ≥14 to <15 °C | ≥15 to <16 °C | ≥16 to <17 °C | ≥17 °C |
| "Average" temperature scenario (2005) | 14.3                                  | 11                                     | 13            | 3             | 3             | 1      |
| "Low" temperature scenario (2006)     | 13.0                                  | 26                                     | 3             | 1             | 1             | 0      |
| "High" temperature scenario (2007)    | 15.2                                  | 6                                      | 8             | 7             | 10            | 0      |

having lasted one and a half time as long as under average conditions. The developmental steps from stages 3 to 4 and stages 4 to 5, respectively, lasted even twice as long as under average conditions. In fact, only very few individuals actually reached the fifth stage, as almost all gravid mussels expelled their larvae prematurely.

The summer of 2007 was, in turn, the summit of an extraordinarily warm period that had already started in September 2006. The water temperature of the River Waldaist never reached the freezing mark in the course of that winter, as it usually does; it actually did not even drop below 2 °C. Especially during the period of egg maturation, water temperatures markedly exceeded average conditions. The mean water temperature in August amounted

to 15.2 °C, 2.2 K more than in the cold summer of 2006. Due to the high temperature, first stage larvae already appeared at the end of June, accordingly four weeks earlier than under average conditions. In fact, reproduction started so early that the first stage had already been completed before the survey was started. The stages succeeded relatively fast, the periods between single stages did not exceed one week each (Fig. 2, bottom). As the water temperature remained higher than average, examinations were continued throughout August. At the beginning of August, a second reproduction cycle started within one single season. Due to the convenient conditions, time spans between the stages were markedly lower than at average temperature terms.



**Fig. 2:** Developmental course of glochidia of a freshwater pearl mussel population in the River Waldaist at different water temperature regimes (top left: "average" mean temperature, 2005; top right: "low" mean temperature, 2006; bottom: "high" mean temperature, 2007). Dots: First occurrence of respective larval stage. Solid line: water temperature in the respective year. Dotted line: water temperature in the average summer of 2005.

Development rates differed markedly between different temperature conditions. The durations of each larval stage and of the entire developmental course depending on the three temperature situations described above are presented in Tab. 3.

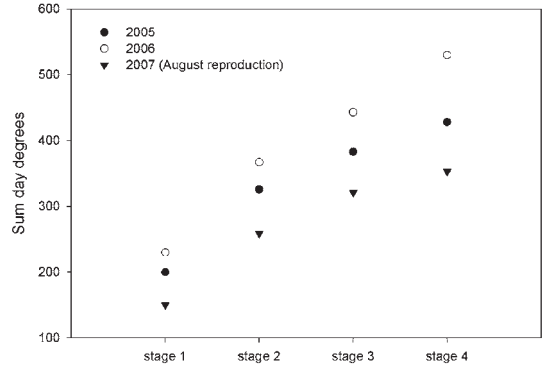
The addition of daily mean water temperatures between the first and the last date of detection of a special stage leads to the degree-days required for the completion of the respective stage (Tab. 3, Fig. 3). The sum of degree-days for the total development ranged from 353 under warm conditions to 530 at low water temperatures. At average terms, the sum of degree-days amounted to 428.

### Discussion

Slight fluctuations in developmental patterns are common in natural systems, as the rate of development in poikilothermic species correlates closely to the temperature of the surrounding medium (e. g. Gordon 1984; Pritchard et al. 1996; Lapointe 2001). Minor temperature shifts between single years have always occurred in the warm-temperate climate of Central Europe. Hence, it can be assumed that developmental cycles in freshwater pearl mussel populations have always varied in length, onset and termination throughout the years, as Hastie & Young (2003) confirm exempli gratia for Scottish rivers. Hence, the different developmental rates in average,

**Tab. 3:** Durations of larval stages and the entire larval development depending on the prevalent water temperature.

|                                       | Duration of development (days) |         |         |         |       | Degree-days |
|---------------------------------------|--------------------------------|---------|---------|---------|-------|-------------|
|                                       | stage 1                        | stage 2 | stage 3 | stage 4 | Total |             |
| "Average" temperature scenario (2005) | 14                             | 9       | 4       | 3       | 30    | 428         |
| "Low" temperature scenario (2006)     | 18                             | 10      | 6       | 6       | 40    | 530         |
| "High" temperature scenario (2007)    | 10                             | 7       | 4       | 2       | 23    | 353         |



**Fig. 3:** Degree-days required for the completion of each developmental stage (cumulative graph). Stage 5 is not included, as it complies with the completion of stage 4.

warm and cold summers presented in the paper at hand are to be considered as natural fluctuations. But the fact that the surveyed population in the River Waldaist performed two complete consecutive reproduction cycles within one single vegetation period in 2007, although the freshwater pearl mussel is regarded strictly univoltine, must be discussed as an absolutely special case. According to the Austrian Central Institute for Meteorology and Geodynamics (ZAMG), the year 2007 can be characterized as one of the warmest years since recordkeeping began. In eleven consecutive months, temperatures above average were recorded. These conditions seem to have influenced the reproductive behaviour of the observed population. It could be detected that coherent parts of the population initiated the generation of glochidia substantially earlier than the rest. As only gravidity was observed and male specimens were therefore not examined for this issue, it is uncertain whether the whole population had split into two separate and independent reproduction groups, or if males generated spermatozoa twice consecutively. Furthermore, the effects of fragmentation into separate reproduction groups with different timing are difficult to estimate. Biological cycles have evolved over long periods of time, always influenced by the prevailing abiotic parameters. In the reproduction cycle of *M. margaritifera* correct timing is a critical success factor. Larvae develop inside their cysts according to the temperature of the surrounding water. As soon as the sum of day degrees reaches a certain value – 1,300 according to Hruška (1998) – metamorphosis takes place and the juvenile mussels drop from



their hosts. Whether temperature conditions in the river are appropriate for juveniles which derive from the early reproduction cycle and therefore drop earlier, cannot be answered without further investigation. In any case, adverse effects on the reproduction success cannot be ruled out. As the IPCC (2001) states that in the near future the temperature will constantly rise, extreme years like 2007 and further asynchronous reproduction cycles are likely to increase in quantity.

Degree-days are a common means for predicting the duration of a developmental stage under certain – often static – circumstances. They are used in aquaculture in order to calculate the time of hatching for fish eggs that are cultivated at a certain, constant temperature (Bishop 1971; Blaxter 1969). However, the exact determination of degree-days for glochidia of the freshwater pearl mussel in the field must be considered almost infeasible. Firstly, in natural habitats the water temperature fluctuates irregularly, depending, inter alia, on the prevailing weather conditions. Furthermore, the significance of mean daily water temperatures also depends on the length of intervals between measurements and is therefore always afflicted with a certain inaccuracy. Moreover, the exact commencement of gravidity can usually not be detected in conventional conservation projects that mostly target on collecting glochidia and normally start at empirically determined dates. As soon as stage 1 larvae can be found, it is impossible to reconstruct the exact time of their original appearance. In warm summers, few days of inaccuracy can lead to perceivable differences in calculated degree-days. The degree-days given in this paper are therefore only approximate values, but are in accordance with Lange et al. (2008) who state a sum of 450 degree-days for the total development of infectious glochidia.

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# A novel system for rearing freshwater pearl mussels, *Margaritifera margaritifera* (Bivalvia, Margaritiferidae), at Mawddach Fish Hatchery in Wales, UK

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## Abstract

In recent years there has been little recruitment in Welsh populations of the freshwater pearl mussel *Margaritifera margaritifera*, and therefore the culture of juvenile mussels in hatcheries is essential to ensure the survival of this species in Wales. Between 2005 and 2008 the first cohort of juvenile freshwater pearl mussels from the Ddu, Eden and Lledr rivers were reared using a novel tank set-up in Mawddach Fish Hatchery, Dolgellau, Wales. For the first seven months juveniles were reared in floating mesh trays with a sparse layer of fine gravel, in flowing tanks with spray bars above. This method supported high survival (> 80%) and growth

of juveniles comparable with other published studies (reaching mean lengths of 0.57 to 0.7 mm after four months). After seven months, juveniles were moved into larger salmonid ova incubation trays with a 1 cm layer of gravel, where they continued to grow slowly, reaching a mean length of 1.4 mm after 24 months, with low survival (0.12%). The floating mesh trays used for the first seven months were easy to build, maintain and monitor, and allowed the rearing of large numbers of juveniles in a small area. These trays are recommended for large-scale rearing of juveniles during the first months post-excystment from fish.

## Résumé

Dans les dernières années il y a eu peu de recrutement au niveau des populations galloises de la moule perlière, *Margaritifera margaritifera*. C'est pour cette raison que l'élevage de jeunes moules perlières est indispensable à la survie de cette espèce au pays de Galles. Entre les années 2005 et 2008 un premier lot de jeunes moules

originaires des cours d'eau Ddu, Eden et Lledr était élevé en utilisant un système de cuve transformée à la pisciculture à Mawddach, Dolgellau, Wales. Pendant les 7 premiers mois les juvéniles étaient maintenus dans des plateaux flottant à mailles dont le fond était couvert d'une fine couche de gravier et au-dessus desquels étaient

suspendus des vaporisateurs. Ces plateaux disposés dans des cuves étaient traversés par un courant d'eau. Cette méthode a permis d'arriver à un taux de survie élevé (> 80%) et à une taille comparable à celle citée dans d'autres études publiées (des longueurs moyennes de 0,57 à 0,7 mm après 4 mois d'élevage). Après 7 mois, les juvéniles étaient transférés dans de plus grands plateaux incubateurs d'oeufs de saumon avec une couche de gravier d'une épaisseur de 1 cm au fond. Dans ce système ils

continuaient à croître lentement pour atteindre une longueur moyenne de 1,4 mm après 24 mois avec un taux de survie bas de 0,12%. Les plateaux flottant à mailles utilisés pendant les premiers 7 mois étaient faciles à construire, à entretenir et à surveiller et ils permettaient d'élever un grand nombre de juvéniles sur une surface réduite. Ces plateaux sont recommandés pour réaliser l'élevage à grande échelle pendant les premiers mois après que les moules se sont détachées du poisson.

### Zusammenfassung

In den letzten Jahrzehnten war das Aufkommen junger Flussperlmuscheln (*Margaritifera margaritifera*) innerhalb der walisischen Populationen sehr gering. Deshalb erscheint die Nachzucht der Flussperlmuschel in Wales unabdingbar, um den Erhalt der Art zu sichern. Zwischen 2005 und 2008 wurden junge Flussperlmuscheln der Flüsse Ddu, Eden und Lledr in einem neuartigen Beckensystem in der Mawddach Fischzucht Station (Dolgellau, Wales) aufgezogen. Die ersten sieben Monate wurden die juvenilen Flussperlmuscheln in Fließrinnen gehältert. Die Jungmuscheln befanden sich dabei in schwimmenden Siebkäfigen in einer dünnen Kiesschicht. Die Siebkäfige befanden sich in einer Fließrinne und wurden gleichzeitig von oben mit Wasser besprüht. Mit dieser Methode wurde eine hohe Überlebensrate der Jungmuscheln erreicht (> 80%) und

das Wachstum der juvenilen Tiere war vergleichbar mit dem Wachstum welches in anderen Studien erzielt wurde (mittlere Länge von 0.57 - 0.7 mm nach 4 Monaten). Nach sieben Monaten wurden die Muscheln in Lachseier-Inkubations-Siebkäfige transferiert, welche eine ein Zentimeter dicke Kiesschicht enthielten. Hier wuchsen die Muscheln langsam weiter und erreichten nach 24 Monaten eine Länge von 1.4 mm. Die Überlebensrate war mit 0.12% allerdings gering. Die Siebkäfige, welche in den ersten sieben Monaten benutzt wurden, sind leicht zu bauen und zu kontrollieren und ermöglichten es eine große Anzahl von Jungmuscheln auf kleiner Fläche groß zu ziehen. Es wird empfohlen diese Methode für die Aufzucht einer grossen Anzahl von Jungmuscheln während der ersten Monate nach der post-parasitären Phase zu verwenden.

## Introduction

The freshwater pearl mussel, *Margaritifera margaritifera* (Linnaeus, 1758), is listed as Endangered on the IUCN Red List (IUCN 2008) and is included in Annex II and V of the EC Habitats & Species Directive. In the UK, it was given full protection on Schedule 5 of the Wildlife & Countryside Act in 1998, and it is listed in the UK Biodiversity Action Plan as a priority species, with the Environment Agency and Scottish Natural Heritage as the lead partners for the Species Action Plan. In response to the lack of recruitment in Welsh rivers, the Environment Agency Wales (EAW) and the Countryside Council for Wales have had a conservation strategy in place since November 2004, and mussels from seven Welsh rivers are currently being held in EAW fish hatcheries in order to collect glochidia to rear juvenile mussels.

North American work on rearing unionid bivalves has demonstrated the importance of flow, food and substrate to juvenile survival (e.g. Hudson

& Isom 1984; Gatenby et al. 1997; O'Beirn et al. 1998; Henley et al. 2001), with different species preferring different combinations of conditions. A variety of methods have been used to rear the margaritifereid *M. margaritifera* in Europe, providing a range of different conditions for juveniles. Intensive methods include holding juveniles at constant temperature in small dishes with regular feeding and cleaning (e.g. Hruška 1999; Lange 2005), and placing juveniles in small cages in rivers (Buddensiek 1995). Less intensive approaches have reared juveniles in natural substrate in an artificial stream receiving unfiltered river water (Preston et al. 2007; Alan Keys pers. comm.), and released infested fish directly into restored river habitats (Altmüller & Dettmer 2001; Rainer Dettmer pers. comm.). All these approaches have supported juvenile survival for more than four years (Buddensiek 1995; Hruška 1999; Rainer Dettmer pers. comm.; Alan Keys pers. comm.). In an artificial stream in Northern Ireland, 10-year-old juveniles have reached 50 mm in length (Alan Keys pers. comm.), and reproducing

populations are now established in restored river habitats in northern Germany (Rainer Dettmer pers. comm.).

A major challenge for the less intensive approaches is monitoring juvenile survival and growth. When juveniles are allowed to excyst from fish directly into an artificial stream or restored river, monitoring is difficult until they reach a size where they can be found in the substrate, approximately four to six years later. This creates a time lag before the success of rearing can be evaluated, and requires long-term planning and funding of rearing projects. The intensive rearing methods pioneered by Buddensiek (1995) and Hruška (1999) in Germany and the Czech Republic make monitoring possible, but there are limits to the numbers of juveniles that can be reared at one time because of the need to handle and move juveniles into dishes or cages.

In order to overcome these limitations, work on rearing pearl mussels in Welsh hatcheries has focused on rearing several thousand juveniles in trays held in flowing tanks, where they can be more easily monitored than in an artificial stream or restored river, using equipment adapted from fish rearing (ova incubation trays) that requires little day-to-day attention or maintenance. This paper describes this method and the results obtained from the first cohort of juveniles reared between 2005 and 2008 at Mawddach Fish Hatchery, near Dolgellau, in Wales.

## Methods

Glochidia from adult mussels from the Ddu, Eden and Lledr rivers (Fig. 1) were used to infest Atlantic salmon (*Salmo salar*) or sea trout (*Salmo trutta* f. *trutta*) in 2005. 66200 juveniles were collected and transferred to the rearing tanks in June 2006, consisting of 28500 Ddu juveniles, 3200 Eden juveniles and 34500 Lledr juveniles. Juvenile numbers were estimated visually under a dissecting microscope.

Young juveniles were initially held in floating 150  $\mu$ m mesh trays, 40 cm long by 32 cm wide, with one tray per mussel population (Ddu, Eden or Lledr), in flowing water troughs, 215 cm long and 40 cm wide. The trays contained a sparse layer of sand and fine gravel (median particle diameter 1.7

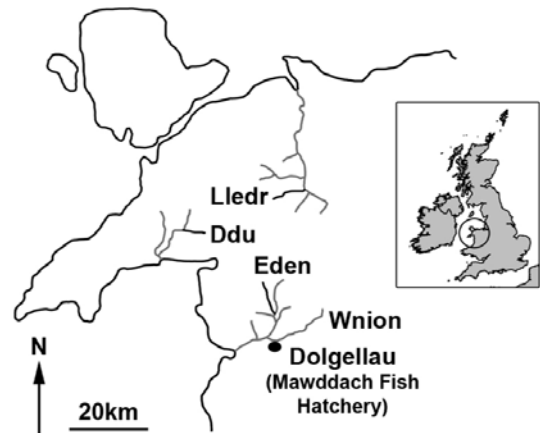


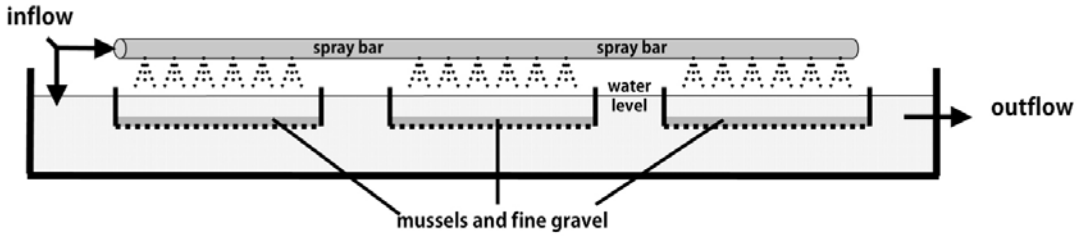
Fig. 1: The source rivers for mussels, also showing the Wnion river, whose water feeds Mawddach Fish Hatchery at Dolgellau.

mm, with 80% of particles between 0.35 mm and 5.6 mm diameter, measured using dry sieving and a Malvern Mastersizer 2000 laser particle sizer). The sediment covered only part of the mesh, and some areas of mesh were clear. The trays received water from below as it flowed underneath the floating trays. The water entering the tanks was filtered through a series of green nylon scourers (suitable for use in the kitchen) and a 400  $\mu$ m mesh. Flow through the troughs was 6 l/min.

On 25 August 2006 some refinements were made to this set-up, shown in Figures 2a and 3. Each tray of juveniles was divided into three similar trays per river population. The water was filtered through a trickle tower, consisting of several trays containing nylon scourers with two 400  $\mu$ m mesh trays at the bottom. In addition to the water flowing through the troughs, water was supplied from above through spray bars, with a flow of 5 l/min going into three mesh trays.

In January 2007, the 7-month-old juveniles were moved to larger standard Californian salmonid ova incubation trays (Fig. 2b). These trays had mesh size 400  $\mu$ m, and were 75 cm long, 39 cm wide and 13 cm deep. They are designed to create an upwelling flow through the mesh because they are shaped so that their back edge blocks off the flow beneath them. More sediment was added to a depth of 1cm, consisting of the original sand and fine gravel mixed in equal quantity with pea gravel (0.5-1 cm diameter). The spray bars were removed at this time and a small sand filter was

**a) Young juvenile set-up**



**b) Older juvenile set-up (8 months +)**

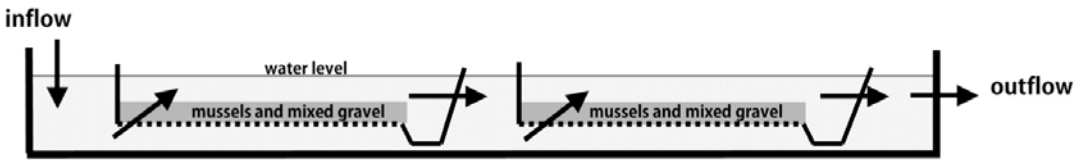


Fig. 2: The tank set-ups used to culture juvenile mussels.

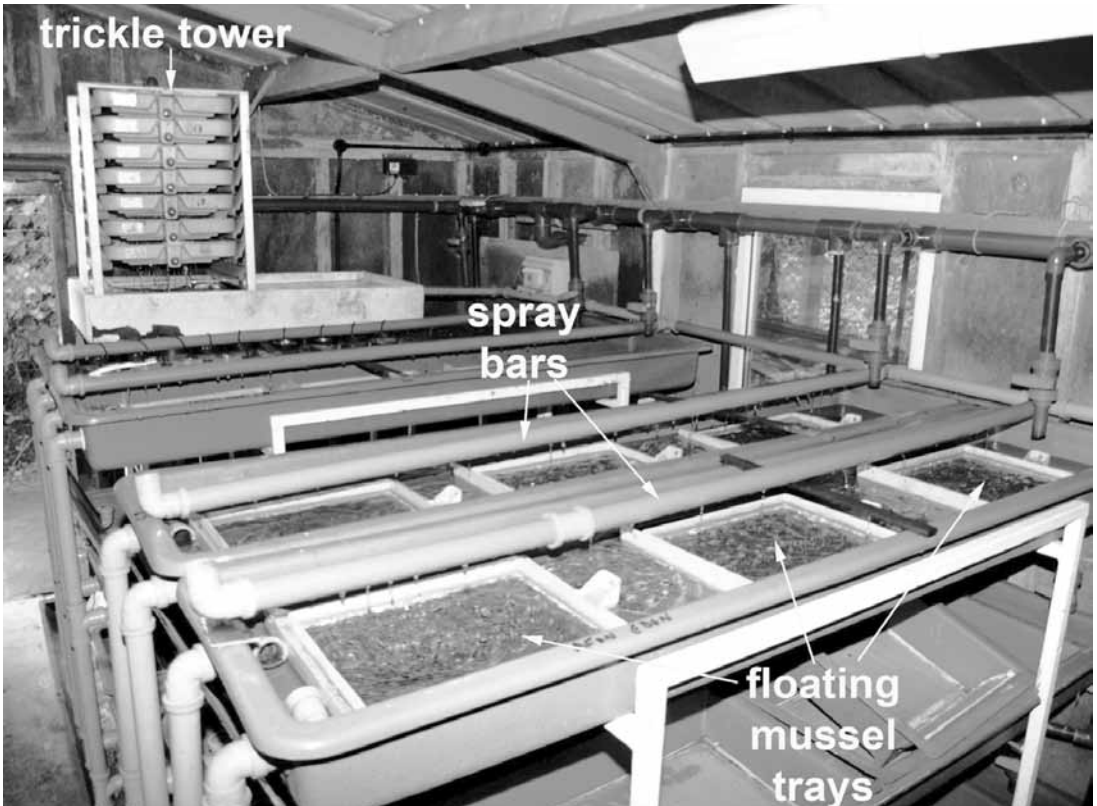


Fig. 3: Early juvenile set-up used from August 2006 to January 2007.

used to filter incoming water after it had passed through the nylon scourers. The water up-welled through the sand filter, which was 45 cm by 60 cm with a 30 cm layer of sand.

At the end of March 2007, the mesh size of the trays was changed to 750 $\mu$ m, and the juveniles were again split so that there were 6 trays per river population. In April 2008 the sand filter was exchanged for a Hydrotech drum filter (Model 801) with a 30  $\mu$ m mesh screen.

The floating mesh trays used initially were not cleaned as the pores remained clear of algae and sediment, allowing a good flow of water past juveniles. After January 2007, the trays were cleaned by draining them down and gently hosing the gravel to remove algae and fine particles from within the sediment, in order to increase the flow of water past juveniles. This was initially carried out fortnightly, but was increased to twice a week by April 2007 because the pores in the sediment quickly became blocked.

All juveniles were held in a building with a small amount of natural light with water supplied directly from the local Wnion river. They were subject to a natural photoperiod and seasonal changes in water temperature. The pH of water entering the hatchery ranges from 6.8 to 7.7 (mean 7.2); dissolved oxygen saturation ranges between 79% and 137% (mean 97%); Biochemical Oxygen Demand (ATU) is less than 1 mg/l and suspended solids (at 105 °C) are less than 3 mg/l throughout the year (data based on monthly measurements throughout 2008 from a sampling point upstream of the hatchery).

Subsets of juveniles were monitored in October 2006 (growth only; 20 Lledr and Ddu juveniles and 10 Eden juveniles), February 2007 (survival only), April 2007 (growth of 26 Ddu juveniles), August 2007 (growth and survival of all Lledr juveniles), and June 2008 (growth and survival of all remaining juveniles). Sub-samples of sediment were removed from trays and sorted under a dissecting microscope. Mussels were counted as live when flesh was visible between the valves, and they could be seen extending their foot. Juvenile lengths were measured using a graticule under a dissecting microscope. In February 2007, the number of surviving juveniles was estimated by dividing each tray into 25 squares and removing a plug of sediment (4 mm in diameter) from a

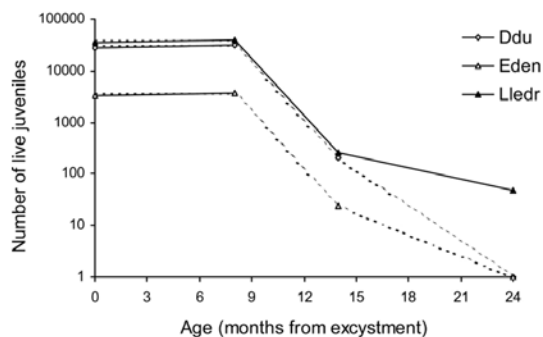


Fig. 4: Survival of juveniles in the first two years post-excystment from fish. Initial estimates of juvenile numbers were lower than estimates after 8 months; as numbers cannot have increased during this time, a dashed line representing 0% mortality is shown for this period. After 8 months, survival of Ddu and Eden populations was not monitored until 24 months, when none remained. The dashed lines show the likely decline in numbers over this period. Note the logarithmic scale on the y-axis.

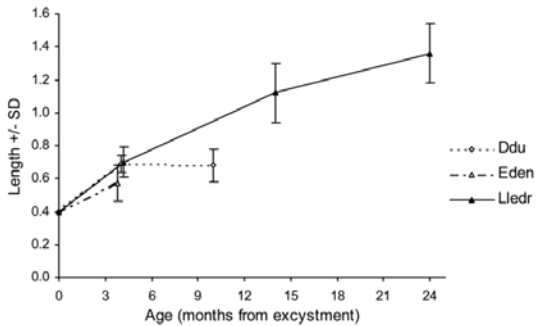
random location within each square; the sediment was divided up into smaller samples for sorting and counting.

## Results

Juvenile survival was very high over the first 8 months post-excystment, and is likely to have been greater than 80% (Fig. 4). The exact survival is not known because of difficulties making visual estimates of juvenile numbers immediately post-excystment from fish; consequently, numbers of juveniles appeared to increase over this period, implying that the initial estimates of juvenile numbers were too low. Between 8 months and 24 months post-excystment, juvenile survival declined dramatically, with 0.65% of Lledr juveniles surviving at 14 months (259 juveniles), and 0.12% at 24 months (47 juveniles) (Fig. 4).

Juveniles from all rivers grew during the first 4 months post-excystment (Fig. 5). Ddu and Lledr juveniles grew significantly more than Eden juveniles, reaching mean lengths of 0.69 mm (Ddu juveniles), 0.70 mm (Lledr) and 0.57 mm (Eden) (ANOVA on lengths at 4 months:  $F_{(2,47)} = 8.8$ ,  $p < 0.001$ ; Tukey's pairwise comparisons showed that Ddu and Lledr juvenile lengths were not signifi-





**Fig. 5:** Growth of juveniles from the different rivers, showing the difference in growth rates between rivers after 4 months (data points have been staggered slightly to increase clarity).

cantly different from each other, but were larger than Eden juveniles; Fig. 5). After 4 months, only Lledr juveniles continued to grow, reaching a mean length of 1.1 mm (maximum length 1.8 mm) after 14 months, and 1.4 mm (maximum length 1.8 mm) after 2 years. The mean length of Ddu juveniles decreased between 4 and 10 months; this probably reflects the lower survival of larger juveniles.

## Discussion

The conditions used to rear young juveniles from June 2006 to January 2007 supported very high survival of juveniles from all three river populations. Factors contributing to this success are likely to include the continuous flow of well-oxygenated water past the juveniles, and the provision of a fine layer of sediment to trap food particles and for juveniles to burrow into. Few data are available to compare survival with other rearing methods: Buddensiek (1995) reports 50% juvenile survival after 4 months in perspex cages in German rivers, and Hruška (1999) found that 10 - 20% survived the period between 2 and 14 months. Our results demonstrate that high survival of large numbers of juveniles can be maintained for several months post-excystment from fish, using simple small-scale rearing systems made from equipment and materials readily available in fish hatcheries. The systems require minimal maintenance and cleaning, and juveniles can be easily monitored by taking sub-samples from within the trays.

Juveniles reached mean lengths of 570 to 700  $\mu\text{m}$  after four months. This is less than that recorded by Buddensiek (1995), when juveniles reached a median length of 700 - 800  $\mu\text{m}$  after 3 months. Higher growth rates have been achieved using intensive laboratory rearing methods at higher temperatures and with additional food (Hruška 1999; Lange 2005; juveniles reached 1.1 - 1.4 mm in length after 3 months). Increasing food availability has been used to increase survival and growth of North American unionid species reared in captivity (e.g. O'Beirn et al. 1998; Jones & Neves 2002), and this is an area for future research at Dolgellau hatchery.

After 8 months, survival decreased; therefore the transfer of juveniles to mesh trays with a deeper layer of sediment is not recommended at this stage. The Californian salmonid ova incubation trays quickly became clogged up with silt and algae, reducing flow past juveniles and probably reducing their supply of oxygen and food. Hosing down the sediment increased flow, but may have caused too much disturbance to juveniles, possibly breaking their fragile shells. In future older juveniles will be transferred to an artificial stream fed with natural river water similar to that used in Ballinderry Fish Hatchery in Northern Ireland (Alan Keys pers. comm.).

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# Growing factors of juvenile freshwater pearl mussels and their characteristics in selected pearl mussel habitats in Saxony (Germany)

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**Keywords:** *Margaritifera margaritifera*, post parasitic stages, semi-natural breeding, bioindication, young mussel ecology, Saxony

## Abstract

Most of the central European *Margaritifera margaritifera* (L.) populations are damaged and close to extinction. In the Czech Republic, a way of semi-natural breeding was developed by Hruška (1999) in the Šumava National Park area in the late 1980s.

In Saxony, the local adaptation of this method was supported by the European funding instrument INTERREG from July 2001 up to December 2007. During that period, the ecology of different life stages after glochidial release could be investigated. The habitat conditions in the saxonian streams are not optimal for development of young mussels and especially seed

mussels. Siltation on the one hand and low quality of natural food on the other hand, as well as age-specific food requirements must be considered.

The development and use of different methods of biological indication allowed food conditions in former and actual mussel brooks and their tributaries to be monitored. For these investigations seed mussels (first summer = 2006) and young mussels up to 18 month old (second summer = 2007) were used. Physical and chemical parameters of local saxonian streams and the Lutter river, were compared.

## Résumé

En Europe centrale la plupart des populations de *Margaritifera margaritifera* (L.) sont menacées et proches de leur extinction.

Fin des années 80 du vingtième siècle une méthode d'élevage semi naturel a été développée par Hruška (1999) dans le Šumava National Park.

En Saxe, l'adaptation locale de cette méthode fut financièrement soutenue par l'instrument européen Interreg de juillet 2001 à décembre 2007. Pendant cette période l'écologie de différents stades de vie des glochidies post-larvaires a pu être examinée. Les conditions de l'habitat des cours d'eau en Saxe sont insuffisantes au développement des jeunes moules perlières et surtout

aux jeunes moules qui viennent de se détacher du poisson hôte. D'une part le colmatage du substrat par des sédiments fins et d'autre part la basse qualité de la nourriture doivent être considérés, de même que les besoins spécifiques en nourriture selon l'âge des moules.

Le type de nourriture présent dans des cours d'eau historiques et actuels de moules perlières a pu être suivi par le développement et l'utilisation de différentes méthodes de bioindication. Pour cette étude des jeunes moules fraîchement détachées des poissons (premier été =2006) et des jeunes moules âgées de 18 mois (deuxième été =2007) ont été utilisées. Les paramètres physico-chimiques de cours d'eau locaux en Saxe et du cours d'eau Lutter ont été comparés.



### Zusammenfassung

Die meisten der mitteleuropäischen Populationen von *Margaritifera margaritifera* (L.) sind beeinträchtigt und kurz vor dem Erlöschen.

In der Tschechischen Republik wurde in den 1980er Jahren im Gebiet des Šumava National Parks ein Weg der halbnatürlichen Nachzucht durch Hruška (1999) entwickelt.

In Sachsen wurde diese Methode durch das Europäische Förderinstrument INTERREG unterstützt und im Zeitraum Juli 2001 bis Dezember 2007 adaptiert. Während dieser Zeit konnte die Ökologie der postparasitären Entwicklungsstadien besser erforscht werden. Die Habitatbedingungen der sächsischen Gewässer sind nicht optimal für die Entwicklung der jungen

Muscheln, insbesondere nicht für die frisch vom Wirtsfisch gefallenen Tiere. Verschlammung und die geringe Qualität der natürlichen Nahrungsressourcen, sowie altersspezifische Anforderungen an die Nahrung spielen hier eine Rolle.

Die Entwicklung und Anwendung verschiedener Methoden der Bioindikation gestattete die Betrachtung der Nahrungsressourcen in früheren und derzeitigen Perlmuschelgewässern sowie ihren Zuflüssen. Für diese Untersuchungen wurden frisch abgefallene Jungmuscheln (erster Sommer = 2006) bis zum Alter von 18 Monaten (zweiter Sommer = 2007) herangezogen. Physikalische und chemische Parameter der sächsischen Gewässer werden mit dem Fluss Lutter verglichen.

## Introduction

In Germany, possibly only one functional population of pearl mussels, in the river Lutter, Lower Saxony survives. Why is the pearl mussel juvenile development in the Lutter so successful? Why not in Saxony and elsewhere?

Which kinds of habitat factors are determining the further development after the parasitic stadium? Apart from the up to date knowledge of the water quality as a need for primary survival, there is need to investigate in the first instance:

- the range of food for growing in general,
- the level of temperature for speed of growing,
- the quality of sediment as the main detrimental factor within the first years (Geist & Auerswald 2007, Altmüller & Dettmer 2006).

## Study area

The investigated area is situated in the south-western part of Saxony: the Vogtland region. Pearl-fishing took place in the catchment area of the "Weiße Elster" river in the past. Less than 80 individuals of this population remained. The main saxonian stock belongs to the catchment area of the "Saale" river and is situated at the czech-german border. With about 1.500 adult pearl mussels this population belongs to the group of

the "Fichtelgebirge" (Nagel in LfULG 2009: 13). Both represent sub-populations of the main Labe population (Geist & Kuehn 2005).

Thin sediment layers of silicic origin are characteristic for both catchment areas. The pattern of the gravel is flat and mostly elongated; pieces of different silicates mixed with minerals and quartzite inclusions in very inhomogeneous sizes.

Water is very poor in calcium and all places belong to the brown trout-region with *Astacus astacus*, *Cottus gobio*, *Lampetra planeri* being co-specific with the common host fish *Salmo trutta fario* (and perhaps *Salmo salar* in the past).

Land use consists of forestry, meadows and farmland in co-existence since the middle ages. Most important is the change in land management. Instead of extensive used grassland we find intensive pasture management close to the river bank or fallow land without any management at all. Both result in changes of vegetation structure and the natural hydrology of wetlands and spring areas.

Most problematic is the land use change in nature protection areas as a result of the lack of management in the course of nature conservation and the difficulties to start coordinating and realizing required measures.

The investigations were mainly made at:

- the border brook Wolfsbach/Bystrina (B), Saxony/Czech Republic without young mussels (incl. the renatured branch A)

- the former pearl brook Haarbach (C), Saxony with a few thousand young mussels in cages (ex "Weiße Elster" and "Saale" both belonging to the Labe system).

The land surrounding C is dominated by *Crepidionetum acutiflori*. The more extensively used land beside the brooks A+B is characterised by *Junco Molinietum caeruleae* with *Phalaris arundinacea* or respectively *Calthion /Phalaris arundinacea*.

Concerning the submergent vegetation we find *Fontinalis antipyretica* as the normal *Callitriche* cf. *hamulata* at locality C as a rare species, whereas at site A and B different *Carex* spec., *Sparganium emersum* and dense *Potamogeton polygonifolius* are present.

Concerning the comparative population of Lutter/Lower Saxony, a functional reference population with many thousand free growing- up young mussels, see paper by Altmüller & Dettmer 2006 for any further details.

## Methods

### Physical and chemical parameters

Water quality was measured according to standardized methods (german standard methods for the examination of water, waste water and sludge, DIN 38410) for leading parameters. Water samples were taken irregularly and analysed by a laboratory (f.e. conductivity, BOD 5, nitrate, orthophosphate, sulphate).

According to these data from the saxonian Interreg-Project, (LFULG 2009) we were able to revert data from the Lutter-Project (Altmüller, NLOE; pers. com.).

In order to establish comparative evaluation approaches temperatures were measured for several years with data loggers. For the main growing period of young juveniles, a reference period (15<sup>th</sup> May – 14<sup>th</sup> September) was chosen. Daily fluctuations, extremes, accumulative degree days and point of glochidia release were compared as far as possible. Changes in the seasonal cycle were also observed.

### Mussel breeding according to a Czech model

In using the semi-natural breeding method according Hruška (2001) the juveniles were kept after release from the host fish for 90 days at a temperature of 16°C. The mussels were examined regularly and dead or dying individuals were removed. Food and water were replaced at any examination. The food (Fine Particulate Organic Matter= FPOM) originated out of the running water using bottle traps or came out of a ditch (D, marsh area). Before feeding the juveniles the FPOM was optimized with animal protein to achieve the expected growth for a good winter survival which is represented by a length of  $\pm 1$  mm (mm-stage).

After the breeding in the laboratory the mussels were brought out in special cages (hole plates) for further development.

### Biological indication I (ex situ)

To determine which of the three investigated brooks (A, B, C) the food quality are meant to be the most promising, groups of mussels were held in the laboratory and fed with different types of FPOM (non-optimized) originating out of the three mussel brooks (A, B, C) (Fig. 2). From former breeding cycles it is known, that the mm-stage is achieved by using optimized FPOM (D). For this reason D (optimized) was used as reference. Analogical to the described breeding method the juveniles were kept for 90 days at 16 °C. At the beginning as well as after 90 days the shell lengths were measured to capture the growth. Besides shell length general condition and physiological constitution of the juveniles were evaluated. Assessment criteria were vitality, sensitivity and growth. The vitality was estimated by the activity shown as pedal feeding trails on the bottom of the container (Fig. 1) the mussels are kept in. It reaches from very low movement shown as rare trails up to a high activity which can be seen in trails all over the bottom. For evaluating the sensitivity, the rapidity of shell closing as reaction of touching was observed. Occurring mortality in the laboratory has poor significance for assessing food quality because of fungal infection and other reasons.

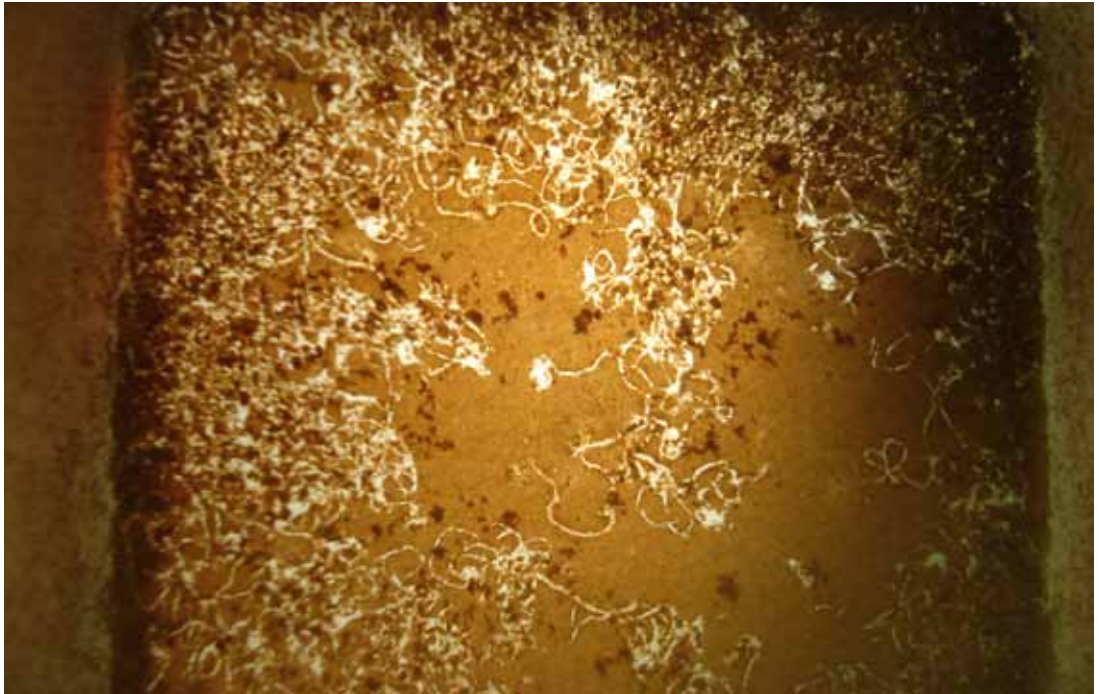


Fig. 1: Trails by pedal feeding mussels in ex situ indication.

Part I (90 days ex situ)

Part II (12 months in situ)

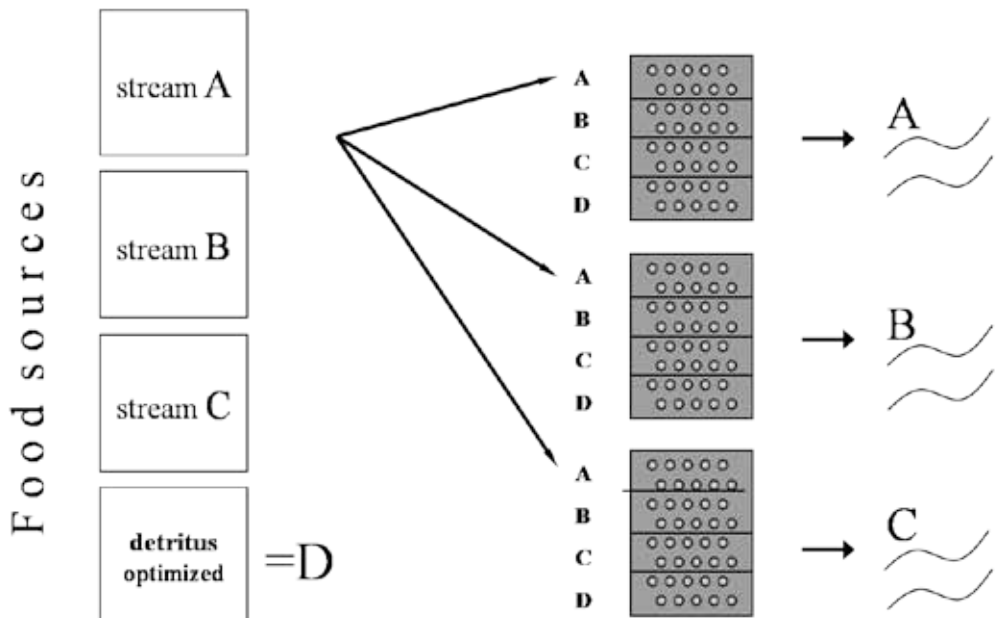


Fig. 2: Schematic diagram of the experiment sequences of biological indication I (ex situ) and II (in situ).

## Biological indication II (in situ)

The biological indication II started after the breeding in laboratory (Fig. 2). The juveniles were caged in hole plates and exposed in the three different brooks (A, B, C). The aim was to determine in which brook the quantity of transported FPOM are meant to be sufficient for further development. Additionally it was investigated to what extend the quality of FPOM which is fed in laboratory is relevant for the further development. In the following 12 months two examinations took place: in May to check the survival rate of winter existence and in September to measure the increase of summer growth. Permanent temperature measuring in the target brooks should allow conclusions about an additional growing factor.

## Statistical analysis

For analysing the increase between the different groups of juveniles the median is used to avoid outliers, all groups were compared with the Kruskal-Wallis Test. Statistical procedures were performed using STATGRAPHICS Plus 4.0.

## Results

### Physical and chemical parameters

Accumulative degree days among the brooks showed a high variance within the reference period (15<sup>th</sup> May – 14<sup>th</sup> Sep.). The highest value was determined with 1942 K in the Lutter followed by brook C with 1771 K and brook B with 1598 K. Maximum

temperatures in the Lutter ranged between 15°C and 20°C and up to approx. 22°C.

Temperatures in brook B ranged between 10°C and 15°C, in brook C around 15°C. In both saxonian brooks temperature values around 20°C were rarely reached. Point of glochidial maturity and release appeared individually according to the different brooks.

Water quality (Tab. 1) showed no significant difference between the Lutter and the two saxonian streams that could explain the advantages of young mussels in the Lutter. Merely the BOD5 showed lower values. Conductivity is controlled by the geology of the catchment area and comparing not thought to be useful.

## Biological indication I (ex situ)

After 90 days clear optical differences (shell colour) (Fig. 4) as well as statistical significant differences ( $p < 0,05$ , Kruskal-Wallis-test) (Fig. 5) according to growth could be determined (Fig. 4). Assessing general condition and physiological constitution differences between B and the other groups were observed. The juveniles of A showed a good sensitivity, the ones of C and D a very good sensitivity, e.g. the shell closed immediately on touch. Individuals of B were mostly passive, 40 % of them died in the 12<sup>th</sup> week. Mortality within the other groups occurred rarely.

## Biological indication II (in situ)

The examinations in May and September (Fig. 6) showed in brook C a high survival rate of individuals and also the highest growth. Survival

**Tab. 1:** Chemical parameters of the Lutter and the saxonian brooks.

| parameter           |       | Lutter                                  | B - Wolfsbach | C - Haarbach  |
|---------------------|-------|-----------------------------------------|---------------|---------------|
|                     |       | Longstanding means (standard deviation) |               |               |
| conductivity (25°C) | µS/cm | 188 (±25,2)                             | 188 (± 31,2)  | 114 (± 18,9)  |
| BOD 5               | mg/l  | 1,08 (±0,43)                            | 2,4 (±0,95)   | 2,3 (±1,17)   |
| NO <sub>3</sub> - N | mg/l  | 2,2 (± 1,1)                             | 2,1 (± 0,78)  | 2,5 (±0,83)   |
| oPO <sub>4</sub>    | mg/l  | 0,042 (±0,03)                           | 0,045 (±0,16) | 0,035 (±0,03) |
| SO <sub>4</sub>     | mg/l  | 31,6 (±6,02)                            | 33,4 (±5,7)   | 22 (±5,1)     |



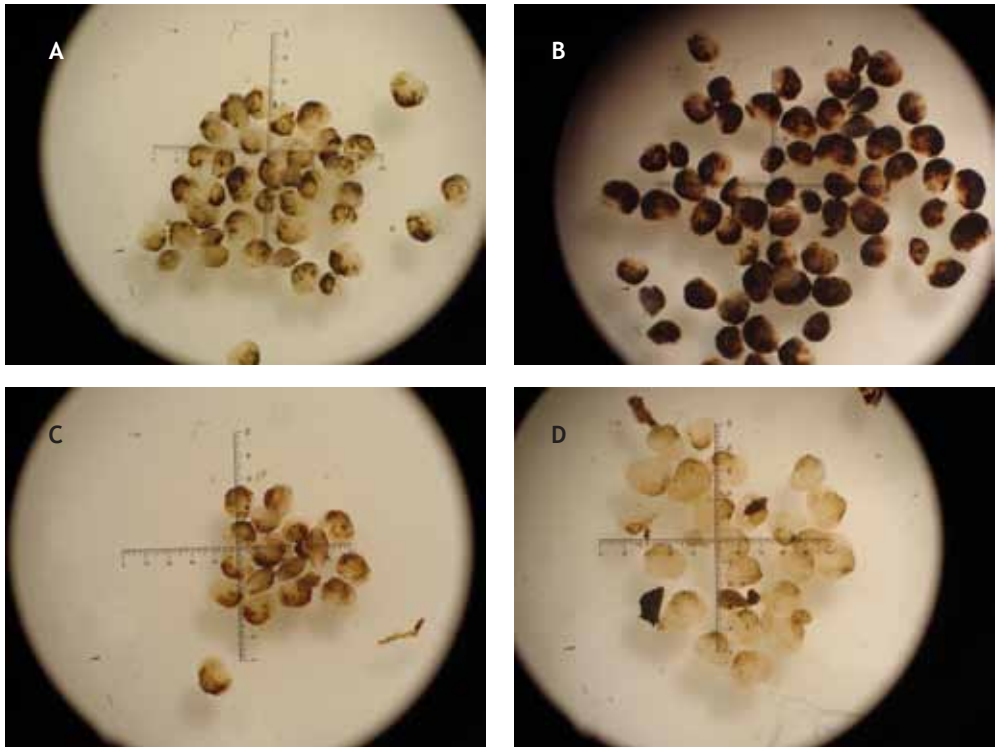


Fig. 3: Optical appearance of the four groups after Biological indication I.

rates and increases were very low in brooks A and B. Main criteria for assessment were the survival rate. In general, survival was related to growth. According to the growth of individuals, the four groups can be ranked as follows: D>C>A>B. The same ranking is also given in brooks A and B.

The individuals fed with FPOM C and D (in Biological indication I) showed the highest survival rate and increase in all brooks.

### Conclusion

Comparing water quality of the Lutter and local (former) saxonian mussel brooks, the conclusion is, that the quality standard is very similar, but still far from that required (for a summary see Sachteleben et al. 2004).

Accepting this suboptimal background, we applied the techniques of the semi-natural breeding (Hruška 1999, Hruška 2001) and of

biological indication (Buddensiek 1995) to study the growing factors food and temperature; both ecological factors are often controversially discussed and possibly misunderstood.

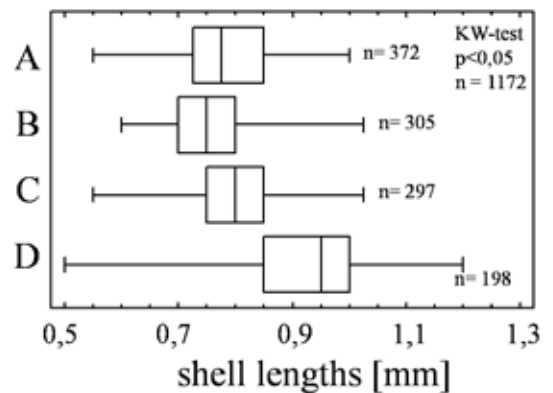


Fig. 4: Distribution of shell lengths within and between the four groups.

| Stream \ Source | A        |              | B        |              | C        |              |
|-----------------|----------|--------------|----------|--------------|----------|--------------|
|                 | Survival | S + Increase | Survival | S + Increase | Survival | S + Increase |
| A               | +/-      | -            | -        | --           | +        | +/-          |
| B               | -        | --           | --       | /            | +/-      | +/-          |
| C               | +        | +/-          | +        | -            | ++       | +            |
| D               | +        | +            | +        | +/-          | ++       | ++           |

**Fig. 5:** Results of Biological indication II (survival(s) = examination in May, S + Increase = Examination in Sept., ++: s > 90%, +: s > 70%, +/-: s > 50%, -: s > 30 %, --: s > 5-1%, /: no survival).

It seems that those juveniles which were fed with high quality food in the first months have an advantage of those fed with food of lower quality.

Bioindicatoric tests determining the availability of natural food (FPOM) are very useful to assess the chances of surviving and of success for culturing and releasing young mussels to the wild.

The results of biological indication II in situ show clearly, that the quality of food is the most important factor for the growth of young pearl mussels (if water quality is acceptable). Sediment quality was not taken into account, as cages were cleaned regularly.

In general it can be confirmed that:

- good water quality is the requirement for survival of juveniles, the water quality in the investigated saxonian brooks allows the survival of young juveniles
- food quality and temperature are essential growing factors. Temperature sums around 1500+x K in the reference period of four summer months is essential for positive growing possibilities

- assessment of good or bad food contents are not yet possible, but it is indicated, that a component of animal protein is indispensable within the first growing period
- natural development of fresh juveniles seems only possible in brook C (Haarbach), but sediment characteristics i.e. siltation clearly limit the chances for development at this site.



**Fig. 6:** Young pearl shell aged 18 month (September 2007/ in situ indication D in C).



The results given in this paper appear to be important for the further discussion of the young *M. margaritifera*'s ecology. It may give assistance in finding strategies to save small populations and to improve chances of any restocking activities.

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# In vitro culture of parasitic glochidia of four unionacean mussels

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**Keywords:** *Anodonta anatina*, *Anodonta cygnea*, *Margaritifera margaritifera*, *Pseudanodonta complanata*, glochidium, artificial culture

## Abstract

Glochidium larvae of four unionaceans were cultured in artificial media. Metamorphosis was achieved in 9 days for *A. anatina* and *P. complanata*, in 13 days for *A. cygnea*, but not for *M. margaritifera*. Result indicates that *in vitro* techniques are most practical in mussels having short parasitic stage with low or no glochidial growth, such as *Anodonta* and *Pseudanodonta*. Mean metamorphosis success of cultured *A. anatina* glochidia was higher in spring cultures (100%) than in autumn (75%), which suggests that the maturity of glochidia increases towards spring, the natural period of glochidia release. Metamorphosis success of *A. cygnea* varied from 32 to 58%, increasing with fungicide concentration. Mean metamorphosis success of *P. complanata* was 43%. Juvenile *A. anatina* originating from the cultivated glochidia were successfully cultured further in the laboratory and grew from 350 to 700-750  $\mu\text{m}$  when fed

with *Scenedesmus* spp. during the 50 days monitoring. For *M. margaritifera* we tested a novel method in which glochidia were cultured with gills of the host fish. Juvenile brown trout, *Salmo trutta*, were first infected with naturally shed glochidia (mean  $\pm$  s.e. length  $67 \pm 1 \mu\text{m}$ ). After 133 days at 8 °C, the gills of infected fish were dissected and transferred to culture medium. The gill-cultured glochidia of *M. margaritifera* metamorphosed to the juvenile stage within 14-15 days (cumulative number of degree-days 1,245-1,267 and the mean  $\pm$  s.e. length  $320 \pm 10 \mu\text{m}$  at metamorphosis). Using gill-culture, we demonstrated the first successful metamorphosis of *M. margaritifera* glochidia in artificial culture medium. Thus, the gill-culture technique may provide a valuable method for studying the host-parasite interaction, maturation and excystment process of *M. margaritifera* glochidia.

## Résumé

Les larves glochidiales de quatre espèces d'*Unionidae* étaient cultivées dans un milieu artificiel. Après 9 jours la métamorphose était achevée pour *A. anatina* et *P. complanata*, après 13 jours pour *A. cygnea*. Ceci

n'était pas le cas pour *M. margaritifera*. Les résultats montrent que les techniques de culture *in vitro* sont le mieux praticable pour des moules qui ont une phase parasitaire de courte durée et une faible croissance

des glochidies ou pas de croissance comme *Anodonta* et *Pseudanodonta*. Le taux moyen de métamorphose des glochidies cultivées de *A. anatina* est plus élevé pour les cultures du printemps (100%) que pour celles de l'automne (75 %). Le taux de maturation des glochidies augmente au printemps, qui correspond à la période naturelle de détachement des glochidies. La réussite de la métamorphose de *A. cygnea* varie entre 32 à 58 % et décroît en fonction de la concentration du fongicide. Le taux moyen de la métamorphose de *P. complanata* était de 43%. Les juvéniles de *A. anatina* originaires de glochidies cultivées étaient élevés avec succès quand elles étaient nourries pendant 50 jours avec *Scenedesmus* spp.. Pour *M. margaritifera* nous avons testé une nouvelle méthode pour laquelle nous avons cultivé les glochidies à l'aide des branchies de poissons.

### Zusammenfassung

Glochiden von vier unioniden Arten wurden in einem künstlichen Medium kultiviert. Eine Metamorphose der Tiere wurde für *A. anatina* und *P. complanata* nach 9 Tagen erreicht und nach 13 Tagen für *A. cygnea* jedoch nicht für *M. margaritifera*. Die Resultate zeigen, dass in vitro Techniken für Arten mit kurzer parasitische Phase und keinem oder geringem Wachstum während dieser Phase möglich sind wie z.B. für *Anodonta* und *Pseudanodonta*. Der mittlere Erfolg der Metamorphose für *A. anatina* war höher für die Gruppe welche im Frühjahr kultiviert wurde (100%) als für die Gruppe die im Herbst kultiviert wurde (75%). Es wird angenommen, dass der Reifegrad der Glochidien im Frühjahr, dem natürlichen Zeitpunkt der Glochidienabgabe, zunimmt. Der Erfolg einer Metamorphose für *A. cygnea* schwankte zwischen 32 und 58% und stieg mit einer Erhöhung der Fungizidkonzentration im Medium. Der mittlere Erfolg der Metamorphose für *P. complanata* lag bei 43%. Juvénile *A. anatina*, welche im Medium aus Glochiden hervorgehen, konnten im Labor erfolgreich für weitere 50 Tage

Les jeunes truites, *Salmo trutta*, étaient soumises à un parasitage avec des glochidies naturellement relâchées (mean  $\pm$  s.e. length  $67 \pm 1 \mu\text{m}$ ). Les glochidies restaient sur les branchies des poissons pendant 133 jours à 8°C. Après cette période les branchies étaient disséquées et transférées dans le milieu de culture. Les glochidies de *M. margaritifera* cultivées sur les branchies des poissons se sont transformés en juvéniles après 14 à 15 jours (cumulative number of degree-days 1,245-1,267 and the mean  $\pm$  s.e. length  $320 \pm 10 \mu\text{m}$  at metamorphosis). A l'aide de la méthode de la culture sur les branchies nous étions les premiers à cultiver avec succès *M. margaritifera* dans un milieu artificiel. La culture de glochidies sur les branchies peut fournir une méthode valable pour étudier l'interaction hôte parasite, la maturation, le désenkystement.

gehältert werden. Sie wurden dabei mit *Scenedesmus* spp. gefüttert und wuchsen von 350 bis auf 700-750  $\mu\text{m}$  an. Für *M. margaritifera* wurde eine neue Methode getestet, bei der die Glochiden mit den Kiemen des Wirtsfisches im Medium gehältert wurden. Junge Bachforellen (*Salmo trutta*) wurden dabei mit auf natürlich ausgestoßenen Glochidien infiziert (mittlere Länge  $\pm$  SD,  $67 \pm 1 \mu\text{m}$ ). Nach 133 Tagen und bei einer Hälterungstemperatur von 8 °C wurden die Kiemen herausgetrennt und in ein Kulturmedium gegeben. Die auf Kiemen kultivierten Glochidien vollzogen eine Metamorphose innerhalb von 14-15 Tagen. (Kumulative Anzahl von Tages-Garden 1,245 -1,267 und mittlere Länge $\pm$ SD  $320 \pm 10 \mu\text{m}$  bei der Metamorphose). Mit Hilfe der Kiemen-Kultur konnte zum ersten Mal eine Metamorphose von *M. margaritifera*-Glochiden in einem künstlichen Medium erzielt werden. Somit könnte die Kiemen-Kultur-Technik dazu benutzt werden, das Wirt-Parasit-Verhältnis, den Reifungs- und Ablösungsprozess von *M. Margaritifera* Glochidien auf der Wirtskieme zu untersuchen.

## Introduction

The concept of using artificial culture to enhance mussel production was introduced as early as the 1920s by Ellis & Ellis (1926). After being dissected out of their cysts from the gills, metamorphosis of *Lampsilis fallaciosa* glochidia in physiological nutrient solutions was reported with varying development times. However, the details of their solutions were not published. Isom & Hudson (1982) were the first to describe the methods for in vitro culture of unionacean glochidia without the use of fish host at any time in the development.

Subsequently, the artificial culture technique has been used for glochidia of several freshwater mussel species (Hudson & Isom 1984; Keller & Zam 1990; Uthaiwan et al. 2001, 2002; Lima et al. 2006). Since many freshwater mussel species have declined over the last decades worldwide (Bogan 1993; Williams et al. 1993), various culturing techniques could potentially be used to produce juveniles for restocking of endangered mussel species (see e.g. Preston et al. 2006).

*Anodonta anatina* (= *A. piscinalis*) is a widespread and abundant mussel inhabiting slowly running waters and littoral zones of temperate lakes in

northern Europe (Bauer et al. 1991). It matures at 2–4 years of age and reproduces annually (Haukioja & Hakala 1978; Bauer 1994; Taskinen & Valtonen 1995), reaching a maximum life span of more than 15 years (Økland 1963; Negus 1966, Haukioja & Hakala 1978). Spawning takes place in early summer and fertilized eggs are stored in the outer gill blades of females, where they develop into glochidia larvae. After release, the glochidia attach to fish for a few weeks, during which they metamorphose (Ellis 1978; Jokela et al. 1991). The glochidia of *A. anatina* can infect several host species (Jokela et al. 1991). After successful metamorphosis, young mussels drop to the bottom, and their benthic life begins.

The natural history of *A. cygnea* and *Pseudanodonta complanata*, is comparable to *A. anatina*, although the spawning of *P. complanata* takes place from May to July (Aldridge 1999) and that of *A. anatina* takes place in June–July (Jokela et al. 1991, Taskinen et al. 1997). The glochidia of *A. anatina* and *A. cygnea* are fully developed in autumn (Jokela et al. 1991) and winter (Cunha & Machado 2001), respectively, whereas maturation of the glochidia of *P. complanata* is more variable (Pekkarinen & Englund 1995). *Anodonta cygnea* attains greater length than the other two species, almost double the length of *P. complanata* (Aldridge 1999).

The freshwater pearl mussel, *Margaritifera margaritifera* L., inhabits pristine small rivers. Like many other unionacean mussels (Bogan 1993; Young & Williams 1983; Williams et al. 1993), *M. margaritifera* has declined dramatically across its entire range of occurrence and the species is now close to extinction in the whole of central Europe (Bauer, 1986, 1988; Gosgrove & Hastie 2001). Glochidia are released by female mussels from June to September (Young & Williams 1984a, 1984b; Hastie & Young 2003). After release to the water they attach to gills of salmonid fish and encyst. The glochidia remain attached to host fish for several months during which they grow and develop into a small mussel, excyst, drop off and start their benthic life, which may last for over 100 years (e.g. Helama & Valovirta 2007).

The problem with many *M. margaritifera* populations is the absence of young individuals (e.g. Bauer 1988; Gosgrove & Hastie 2001). In a number of these otherwise potentially viable populations, no juvenile recruitment has been reported for several decades (Young et al. 2001). In some of

these populations, the mussels are able to produce glochidia that infect fish, but there may be no suitable habitats available for juvenile mussels. Young & Williams (1984a) estimated that only one of 10<sup>8</sup> shed glochidia became settled juveniles. So, survival of *M. margaritifera* glochidia to the juvenile stage is extremely low even in viable populations. Therefore, increasing the survival of juvenile mussels is the key step in conserving *M. margaritifera*.

The aim of the present study was to culture the glochidia of four freshwater unionacean mussels: *Anodonta anatina* and *A. cygnea*, two common species; *Margaritifera margaritifera*, a globally endangered species; and *Pseudanodonta complanata*, a nearly threatened Red Data List species. Previously only glochidia of *A. cygnea* have been successfully cultured artificially (Lima et al. 2006). A further aim was to evaluate the viability of juvenile *A. anatina*, originating from the cultivated glochidia.

## Materials and Methods

### *In vitro* culture of glochidia of *Anodonta anatina*, *Anodonta cygnea* and *Pseudanodonta complanata*

Using scuba, 25 individuals of *A. anatina* and 29 individuals of *P. complanata* were collected from the littoral zone of Lake Leppävesi, near the city of Jyväskylä, southern Finland, in spring (27 April) and autumn (18 September) 2005. Twenty six *A. cygnea* were collected from Lake Uksjärvi, near the city of Pori, southern Finland, on 30 October 2005. Mussels were transported to the laboratory in 60 L containers with lake water. In the laboratory, mussels were dissected and the whole marsupial outer demibranchs with mature glochidia were removed. The gills were cut with sterile forceps and scissors, releasing the larvae into a beaker of sterilised lake water. The glochidia were rinsed to remove all the fragments of parental gill tissue. Glochidia larvae were collected from the beaker using a sterile Pasteur pipette and approximately 100 larvae were placed in each tissue culture dish (width 60 mm, height 15 mm) with 2 ml D-MEM. One ml of new born calf serum was added to the medium. In addition, antibiotics (PSN Antibiotic Mixture, Life Technologies) and fungicide

(Amphotericin B, Life Technologies) were added at concentrations of 100 and 5  $\mu\text{g ml}^{-1}$ , respectively. In *A. cygnea* cultures, concentrations of 6, 7 and 8  $\mu\text{g ml}^{-1}$  of fungicide were tested while keeping the antibiotic concentration at 100  $\mu\text{g ml}^{-1}$ . The tissue culture dishes (30 dishes/species) were kept in a 5%  $\text{CO}_2$  atmosphere in an incubator to maintain the proper carbonate buffer. Another medium, L-15 of Leibowitz designed for incubation without  $\text{CO}_2$ , was used for an additional 60 dishes of *A. anatina* and *P. complanata*. Temperature was kept at 20.0 °C during the cultivation. Culture medium was not changed during cultivation of *A. anatina* and *P. complanata*, but was changed on the 5<sup>th</sup> day during cultivation of *A. cygnea*. Sterilised lake water was added to medium 1:1 between days 7 and 9, and 100% lake water between days 8 and 10.

Viability of glochidia at the start of cultivation was estimated from observations of 3 fields of view using a dissection microscope with 40 $\times$  magnification from randomly selected dishes. Glochidia that closed their valves were considered alive. Our criterion for the successful metamorphosis into the juvenile stage was that the moving foot was clearly operating outside the shell. The mean metamorphosis success, adjusted for the viability of glochidia at the beginning, was estimated as:  $100 \times [\text{proportion transformed during cultivation (\%)} / \text{viability of glochidia at the beginning of the cultivation (\%)}] \%$ . To achieve the assumptions of parametric statistical analyses, arcsine transformations were performed on glochidial viability and metamorphosis success data.

#### **In vitro culture of glochidia of *Margaritifera margaritifera***

We collected 100 pearl mussels on 28 September 2004, by scuba diving from Ähtävänjoki river, western Finland. Mussels were transported to the laboratory in river water and kept in 60 L containers in aerated ground water where they were allowed to release their glochidia. The mussels were then returned to the river. The mussels were collected and handled under licence from the West Finland Regional Environmental Centre, Vaasa, Finland. To adapt the mussels to laboratory conditions, water temperature in the containers was allowed to increase to 20 °C over 36 hrs. The water was inspected microscopically and glochidia were collected with a sterilized 1 ml syringe with a needle no. 13, (0.3 mm

in diameter and 13 mm in length) and placed in 40 culture dishes, 50 glochidia per dish, total of 2000 glochidia (Table 1). Each dish contained 2 ml D-MEM, 1 ml new born calf serum, antibiotics and fungicide as above. The tissue culture dishes were maintained in a 5%  $\text{CO}_2$  atmosphere at 20 °C. The culture medium was changed every 7 days. Growth of glochidia was measured microscopically from a subsample of glochidia every 2<sup>nd</sup> week, but here results are shown only for the start and end points (Table 1).

#### **In vitro culture of fish gills infected with *M. margaritifera* glochidia**

On 4 October 2004, 0+ brown trout (*Salmo trutta*) fry (Ähtävänjoki river stock,  $n = 220$ ) were transported from a fish farm to the laboratory. The next day, the fish were exposed to glochidia shed by the pearl mussels on 28 September 2004 (see above). Infections were completed by placing 4 batches of 55 brown trout in a well-aerated 50 L container with approximately 20,000 glochidia for 20 min. After the exposure, the fish were rinsed and kept in fresh water for 10 min to remove the glochidia from the skin. Thereafter the fish were kept in a 1000 L tank at 8.0 °C in ground water and fed daily with commercial food pellets. Infection and maintenance of fish was done under licence from the Ethical Committee for Animal Research of the Finnish Game and Fisheries Research Institute (Licence no. 13/06).

On 30 November 2004, when the developing glochidia were 56 days old, 11 infected brown trout were killed and the gill arches dissected and cleaned with sterilised river water. We put the gill arches in a total of 22 dishes (2 arches per dish) with culture medium (see above). Group 1 (10 dishes) was kept at 20 °C with  $\text{CO}_2$  and Group 2 (12 dishes) was kept at 1-4 °C without  $\text{CO}_2$  (Table 1). On 24 February 2005, after 87 days of dish culture, when the age of glochidia was 142 days, we divided Group 2 into 2 subgroups, Group 2A and Group 2B, having different temperature and  $\text{CO}_2$  treatments (for details, see Table 1). Culture media were changed every 7 days. When needed, we removed the mucus shed by the gills using a small brush during each medium renewal. We monitored growth of the glochidia by measuring microscopically, by using transmitted light, the length from a sample of glochidia selected randomly from different dishes. In Group 2A and Group 2B, a total of 13 length measurements



were made between the ages of 142 and 230 days. However, the results are shown only for selected time points presented in Table 1. Monitoring of Group 1 was ended on 4 January 2005, on the 34<sup>th</sup> culture day (total age of glochidia 90 days) and monitoring of Group 2A and 2B was ended on 17 May 2005, when the last glochidia died at the age of 230 days, after 174 days of culture.

Group3 was established on 15 February 2005 (age of glochidia 133 days) by dissecting gills of 2 infected brown trout and making 2 culture dishes with 4 gill arches per dish (Table 1). The dishes were kept with CO<sub>2</sub> in 16 °C for the first 9 days and in 22 °C for the next 7 days. Instead of D-MEM, M199 (Earle's salt) was used as the culture medium. Culture media were changed and mucus removed on days 2 and 6. Growth of the glochidia was measured microscopically as above. A total of 8 length measurements were done for Group 3 between the ages of 133 and 149 days. However, the results are shown only for selected time points presented in Table 1. When movement of the foot of the glochidia was observed we added 2 mL of sterilised water from Ähtävänjoki river to the dishes. One day after that, we transferred the gills to sterilised river water without carbonate buffer. Monitoring of Group 3 was ended on the 20<sup>th</sup> culture day, 7 March 2005, when the last metamorphosed juvenile died.

### Culture of juvenile *A. anatina*

After metamorphosis, *A. anatina* juveniles originating from artificially cultured glochidia of the spring cultivation 2005 (see above) were transferred from the dishes gradually to larger water volumes with aeration so that they were in five 500 ml containers by 4 weeks after the metamorphosis. Juveniles were kept without substratum and the water was partly changed every 4<sup>th</sup> day. The green alga *Scenedesmus* spp. was added after each water change in an amount to give a light greenish colour to the water. Growth of juveniles was monitored for 50 days.

## Statistical analyses

Spring and autumn metamorphosis success of *A. anatina* was compared using t-test and the effect of fungicide concentration on metamorphosis success of *A. cygnea* was analysed using 1-way

ANOVA. Differences in the length of *M. margaritifera* glochidia between groups or between different time points within a group were analyzed using t-test. Statistical analyses were performed using SPSS for Windows 14.0 (SPSS inc., Chicago, IL, USA). Measurement means are given with one standard error (s.e.) of the mean.

## Results

### In vitro culture of glochidia of *Anodonta anatina*, *Anodonta cygnea* and *Pseudanodonta complanata*

The mean viability of *A. anatina* glochidia at the start of cultivation was 86.2 ± 1.2% in spring and 77.9 ± 4.2% in autumn, the difference being marginally significant ( $t = 2.063$ ,  $df = 15$ ,  $P = 0.057$ ). The metamorphosis of glochidia to juveniles occurred in nine days. The mean adjusted metamorphosis success of glochidia was 100.0% in spring and 74.6 ± 2.0% in autumn. The metamorphosis success was significantly higher in spring cultivations than in autumn cultivations for *A. anatina* ( $t = 3.175$ ,  $df = 26$ ,  $P = 0.004$ ). The mean viability of *A. cygnea* glochidia at the start of cultivation was 76.1 ± 1.1 in autumn. The metamorphosis of glochidia to juveniles occurred in 13 days. In fungicide concentrations 6, 7 and 8 µg ml<sup>-1</sup>, the mean adjusted metamorphosis success of glochidia was 31.9 ± 3.5, 39.9 ± 2.6 and 57.7 ± 3.9%, respectively. Fungicide increased metamorphosis success statistically significantly (1-way ANOVA,  $F = 14.855$ ,  $df = 2, 87$ ,  $P < 0.001$ ). Post hoc test revealed, that metamorphosis successes did not differ from each other at the lowest fungicide concentrations (6 vs. 7 µg ml<sup>-1</sup>;  $P = 0.373$ ), but differed between the lowest and highest (6 vs. 8 µg ml<sup>-1</sup>) and between the second highest and highest (7 vs. 8 µg ml<sup>-1</sup>) concentrations ( $P < 0.001$ ). Mean viability of *P. complanata* glochidia at the start of autumn cultivation was 58.4 ± 10.7%. Metamorphosis of glochidia to juveniles occurred in nine days and the mean adjusted metamorphosis success of glochidia was 42.8 ± 0.3%. Medium L-15 of Leibowitz was tested for *A. anatina* and *P. complanata*. Glochidia developed properly and inner organs were visible, but larvae failed to metamorphose.



### **In vitro culture of glochidia of *Margaritifera margaritifera***

No bacterial or fungal growths were observed on the culture dishes. Cultivation ended on day 55 when the last glochidia died. The glochidia did not metamorphose into the juvenile stage. The mean size of the glochidia increased (Table 1) significantly during the 55 days ( $t = -3.808$ ,  $df = 1,15$ ,  $P = 0.002$ ). However, when compared to the glochidia originating from the same mussels but being parasitic on fish (Group 1 in Table 1, measured at the age of 56 days) the daily growth rate of cultivated glochidia was only one third that of those on fish gills, and they were significantly smaller (Table 1,  $t = -2.780$ ,  $df = 1,20$ ,  $P = 0.012$ ).

### **In vitro culture of fish gills infected with *M. margaritifera* glochidia**

**Group 1** suffered from profuse mucus secretion by gills and from fungal growth. The gill arches degraded slowly, the glochidia excysted, and the cultivation ended on day 34 (total age of glochidia 90 days). The mean length of the glochidia increased significantly (Table 1,  $t = -5.753$ ,  $df = 1,21$ ,  $P < 0.001$ ). As compared to free, unencysted glochidia cultured at the same temperature (20 °C), or to the glochidia parasitic on fish kept at a lower temperature (8 °C), the daily growth rate of gill-cultured glochidia of Group 1 was ca. 7.4 and 2.5 times higher, respectively (Table 1). However, no metamorphosis to juvenile stage was observed.

**Group 2** did not suffer from mucus secretion or fungal growth. During the 86 days of cultivation (total age of glochidia 142 days) the mean length of glochidia increased significantly (Table 1,  $t = -8.500$ ,  $df = 1,63$ ,  $P < 0.001$ ). The daily growth rate was 1.4 times higher in this group (kept in 1-4 °C) than in glochidia parasitic in fish kept in 8 °C (Table 1). Culturing of Group 2 was continued as subgroups 2A and 2B (results below).

**Group 2A and Group 2B** – Length of glochidia increased significantly in both Group 2A ( $t = -8.079$ ,  $df = 1,70$ ,  $P < 0.001$ ) and Group 2B ( $t = -9.552$ ,  $df = 1,63$ ,  $P < 0.001$ ) during cultivation from an age of 142 to an age of 201 days. However, during that period the daily growth rate of glochidia in Group2B was 1.5 times that in Group 2A (Table 1), and the difference in the length of the glochidia at the end of the period was significant ( $t = -4.198$ ,  $df = 1,35$ ,  $P < 0.001$ ). Between days 201 and 230, the growth in Group 2B slowed down so

that the daily growth rate was finally negative as measured from living glochidia, whereas Group 2A continued their daily growth at the same level as before (Table 1). Therefore, the mean sizes of the glochidia in Group2A and Group2B did not differ at the end of the cultivation ( $t = 0.178$ ,  $df = 1,18$ ,  $P = 0.861$ ) (Table 1). None of the glochidia metamorphosed into juvenile mussels. Inner organs were visible at the end of the cultivation but no movement was observed inside the glochidia.

The glochidia of **Group 3** grew significantly during the first 9 days of cultivation, between 133 and 142 days of age ( $t = -5.289$ ,  $df = 1,65$ ,  $P < 0.001$ ) (Table 1). Meanwhile, the length of the *M. margaritifera* glochidia developed in fish hosts at the same temperature, increased in 7 days from  $270 \pm 7 \mu\text{m}$  ( $n = 16$ ) to  $326.7 \pm 4.8 \mu\text{m}$  ( $n = 36$ ). Thus, the glochidia grew significantly faster in the fish than in the artificial medium (from 270 to 301  $\mu\text{m}$ , Table 1) ( $t = 5.261$ ,  $df = 1, 85$ ,  $P < 0.001$ ). However, the mean daily growth rate of the glochidia during the cultivation at 16-22 °C was ca. 2 times higher than in fish host at 8 °C during the 133 days before the cultivation (Table 1). Overall, the highest growth rates, more than 3  $\mu\text{m day}^{-1}$ , were observed in Group 3 during the artificial cultivation at 16 °C. We observed movement of the foot inside the glochidia on the 10<sup>th</sup> cultivation day when the total age of glochidia was 143 days. The first metamorphosed juvenile *M. margaritifera* was observed on the 14<sup>th</sup> cultivation day, the age of the glochidium being 148 days. Two other juveniles were found on the next day. The mean length of the newly metamorphosed *M. margaritifera* juveniles was  $320.0 \pm 10.0 \mu\text{m}$  (Table 1). The number of degree-days required from the shedding of glochidia till the metamorphosis into a juvenile mussel was 1245-1267. Although metamorphosis was achieved, the size of the developing glochidia was highly variable. Adding sterilised river water into culture dishes induced metamorphosis of largest individuals but killed the smaller, still developing, ones.

### **Culture of juvenile *A. anatina***

The 50 days culture of juvenile *A. anatina* was successful. Juveniles were observed to grow from approximately 350 to 700-750  $\mu\text{m}$  during the 50 days monitoring.

**Table 1:** In vitro culture of free glochidia of *Margaritifera margaritifera* and those encysted in the gills of fish host.

Number of dishes for each culture group, age of glochidia and culturing conditions are given. Length 1 and Length 2 represent the mean  $\pm$  s.e. length at the beginning and at the end of each period. G denotes the daily growth rate ( $\mu\text{m}$ ), and M denotes whether metamorphosis was observed (+) or not (-).

| Group          | No of dishes | Age (days) | Conditions             | Length 1                 | Length 2                 | G    | M |
|----------------|--------------|------------|------------------------|--------------------------|--------------------------|------|---|
| Free glochidia | 40           | 0-55       | 20 °C, CO <sub>2</sub> | 66.5 $\pm$ 0.9 (n = 10)  | 72.5 $\pm$ 1.2 (n = 7)   | 0.11 | - |
| Group 1        |              | 0-56       | fish, 8 °C             | 66.5 $\pm$ 0.9 (n = 10)  | 85.0 $\pm$ 3.4 (n = 15)  | 0.33 |   |
|                | 10           | 56-90      | 20 °C, CO <sub>2</sub> | 85.0 $\pm$ 3.8 (n = 15)  | 112.5 $\pm$ 3.4 (n = 8)  | 0.81 | - |
| Group 2        |              | 0-56       | fish, 8 °C             | 66.5 $\pm$ 0.9 (n = 10)  | 85.0 $\pm$ 3.4 (n = 15)  | 0.33 |   |
|                | 12           | 56-142     | 1-4 °C                 | 85.0 $\pm$ 3.8 (n = 15)  | 124.6 $\pm$ 2.4 (n = 50) | 0.46 |   |
| Group 2A       | 6            | 142-201    | 4 °C                   | 124.6 $\pm$ 2.4 (n = 50) | 154.8 $\pm$ 1.5 (n = 22) | 0.57 |   |
|                | 6            | 201-230    | 19 °C, CO <sub>2</sub> | 154.8 $\pm$ 1.5 (n = 22) | 171.6 $\pm$ 6.9 (n = 8)  | 0.58 | - |
| Group 2B       | 6            | 142-201    | 19 °C, CO <sub>2</sub> | 124.6 $\pm$ 2.4 (n = 50) | 174.2 $\pm$ 5.2 (n = 15) | 0.84 |   |
|                | 6            | 201-230    | 19 °C, CO <sub>2</sub> | 174.2 $\pm$ 5.2 (n = 15) | 171.3 $\pm$ 3.9 (n = 12) | -0.1 | - |
| Group 3        |              | 0-133      | fish, 8 °C             | 66.5 $\pm$ 0.9 (n = 10)  | 270.0 $\pm$ 6.9 (n = 16) | 1.53 |   |
|                | 2            | 133-142    | 16 °C, CO <sub>2</sub> | 270.0 $\pm$ 6.9 (n = 16) | 300.6 $\pm$ 2.4 (n = 51) | 3.40 |   |
|                | 2            | 142-149    | 22 °C, CO <sub>2</sub> | 300.6 $\pm$ 2.4 (n = 51) | 320.0 $\pm$ 10.0 (n = 3) | 2.77 | + |

## Discussion

To our knowledge, this is the first time *A. anatina*, *P. complanata* and *M. margaritifera* glochidia have grown and metamorphosed in an artificial culture medium. However, unlike the other three species cultivated, the metamorphosis of *M. margaritifera* took place only among glochidia cultured with the host fish gills, and not among free, unencysted glochidia. Therefore, with the present method *M. margaritifera* juveniles can probably not be produced without infecting the fish host. As the infected fish were dissected when the age of *M. margaritifera* glochidia was 56 days, the glochidia survived in the artificial medium within the gills up to 174 days (total age of glochidia 230 days), but did not metamorphose. When the gill-culture was started at the age of 133 days, a successful metamorphosis was achieved in 14 days. Thus, the gill-culture method may require substantially advanced development of glochidia within the fish host prior to transfer to artificial culture medium.

Short-term gill filament culture systems have been developed for some fish species (e.g. Bury et al.

1998; Mazon et al. 2004). These types of culture systems eliminate various internal factors (i.e. hormonal and physiological state of fish) which may complicate experiments *in vivo* (Mazon et al. 2004), and they also reduce the number of experimental animals required. The presented gill-culture method for *M. margaritifera* might be improved by using culture medium optimal for fish tissue or by using plasma from the host fish, instead of the calf serum (see Uthaiwan et al. 2002). Nevertheless, the current gill-culture technique may provide a valuable method for studying the host-parasite interaction, maturation and excystment process of *M. margaritifera* glochidium.

The developmental rate and duration of the glochidial stage is temperature-dependent. The glochidia of *A. anatina*, *A. cygnea* and *P. complanata* are relatively large (350  $\mu\text{m}$ ), show low or no growth in the fish host and develop reasonably quickly into the juvenile stage. In contrast, *M. margaritifera* glochidia are small (70  $\mu\text{m}$ ) when shed from the female mussel, and their development in fish takes a long period, 1600 degree-days at 8.0 °C (Bauer 1994) and 1300-1430 degree-days at 15.5-17.0 °C (Hruska 1992), during which

they increase their original size 4-5 fold. The number of degree-days required for metamorphosis of *M. margaritifera* glochidia in the current study was 1245-1267. These life history characteristics may make the in vitro culture of *M. margaritifera* glochidia a demanding task.

In vitro metamorphosis of glochidia has been successful with a number of mussel species (e.g. Isom & Hudson 1982; Keller & Zam, 1990; Roberts & Barnhart 1999, Lima et al. 2006; Uthaiwan et al. 2001, 2002). In previous studies, the metamorphosis of glochidia to juveniles occurred after 9 to 30 days in culture depending on the mussel species, culture temperature (20-26 °C), and glochidial maturity at the start of incubation (e.g. Isom & Hudson 1982; Hudson & Shelbourne 1990, Lima et al. 2006). In the present study, the metamorphosis of glochidia to juveniles occurred in nine days in *A. anatina* and *P. complanata* and in 13 days in *A. cygnea* at 20 C°, which is in accordance with previous results. In the study by Lima et al. (2006), the development to metamorphosis took 11 days in *A. cygnea* at 23 ± 2 C°.

In *A. anatina*, the metamorphosis success was significantly higher at the time of their natural glochidial release in spring than in autumn cultivations. This may result from glochidial maturity. Jones et al. (2005) reported that the survival and growth of juveniles of oyster mussel, *Epioblasma capsaeformis*, were significantly greater when propagated in fish in the spring, when the glochidia were mature and would normally be released, than in fall. Since *P. complanata* and *A. cygnea*, glochidia were cultured only in autumn, metamorphosis success might have been higher in spring, when glochidia are normally released by female mussels of those species.

High in vitro metamorphosis success, > 90%, has been achieved in some earlier studies (e.g. Keller & Zam 1990; Roberts & Barnhart 1999). The present complete metamorphosis success of *A. anatina* glochidia in spring cultivation when 100% of the initially viable glochidia metamorphosed is, to our knowledge, the highest recorded for in vitro cultivations of unionid glochidia. Interestingly, results by Roberts & Barnhart (1999) on *A. suborbiculata* show that a higher proportion of glochidia metamorphose in an artificial culture medium than in the natural fish host, and that this is probably due to absence of fish immune response in the artificial culture medium. Our

results indicated that artificially cultured *A. anatina* glochidia survive and grow after metamorphosis as juveniles in the laboratory. However, the viability and success of juvenile freshwater mussels originating from artificially cultured glochidia should be thoroughly investigated.

## Conclusions

Our study demonstrated that the metamorphosis of *A. anatina*, *P. complanata* and *M. margaritifera* glochidia can be achieved in an artificial culture medium, although for *M. margaritifera* only by the gill-culture method. The results also indicated that these in vitro techniques are most practical for mussel species having a large glochidium and a short parasitic stage (few days) with low or no growth involved, such as *A. anatina*, *A. cygnea* and *P. complanata*. In contrast, glochidia of *M. margaritifera* glochidia are small and their development in fish takes several months during which they increase their original size 4-5 fold, making their artificial culture a challenge. Therefore, with the present method *M. margaritifera* juveniles can probably not be produced without infecting the fish host. Viability and metamorphosis success of *A. anatina* glochidia in artificial culture was higher at the time when they would normally be released, in spring. Our results indicated that artificially produced glochidia of *A. anatina* grow well as juveniles in the laboratory for at least 50 days when fed with *Scenedesmus* spp.. However, viability of the juveniles should be carefully investigated, as well as optimization of the culture media, for example by using fish plasma. In the case of *M. margaritifera*, our novel gill-culture method could be utilized to study the host-parasite interaction, and glochidial maturation and excystment processes of this globally endangered unionacean species.

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# Shell growth and age determination in the freshwater pearl mussel *Margaritifera margaritifera* in Sweden: natural versus limed streams

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**Keywords:** shell growth, *Margaritifera margaritifera*, freshwater pearl mussel, annual growth, growth factor, age - shell length relationship

## Abstract

In most of the European countries there are general efforts to improve the environmental conditions necessary for the regeneration of existing populations of the freshwater pearl mussel, *Margaritifera margaritifera*. In order to create sustainable conservation strategies it is important to have a scientific background for the biology of this species.

In this paper we present general aspects of shell growth in freshwater pearl mussels, mostly from Swedish populations but also from Kola Peninsula. Unique shell material, collected between 1984 and 2006 and donated to the Swedish Museum of Natural History in Stockholm by the governmental authorities in charge of environmental monitoring, gave us the opportunity to study shell growth even in juvenile bivalves. This enabled us to make very precise age estimations of the eroded umbral parts in old shells. The abundant shell

material allowed us to establish the relationship between age and shell length for *M. margaritifera* in Sweden and to construct growth curves that enable a more precise age estimation by measuring the shell length, and in this way to provide a better tool for the monitoring work. However, there is a variation of the shell growth in relation to age between populations in different rivers and we propose three growth curves that approximate the age - shell length relationship: the normal, high and low growth curves. There is no evidence that the age - shell size relationship has a N - S biogeographical gradient, although an annual growth trend related to the temperature has been observed. Also, it has been observed that the annual growth rate of the shells is affected by changes in water quality, for example due to liming. We demonstrate that *M. margaritifera* mussels, especially the juvenile stages, are very sensitive to sudden changes in their environment.

## Résumé

Dans la plupart des pays européens de considérables efforts sont réalisés dans l'amélioration des conditions environnementales nécessaires à la régénération des populations existantes de la moule perlière, *Margaritifera margaritifera*. Pour développer des stratégies de conservation durable, il est important de disposer d'une base scientifique de la biologie de cette espèce.

Dans la présente publication, nous présentons des aspects généraux de la croissance des valves de mollusques d'eau douce. Surtout des populations suédoises sont traitées, mais sont abordées également celles de la péninsule de Kola. Il s'agit de matériel unique de coquilles, collecté entre 1984 et 2006 par les autorités gouvernementales dans le cadre d'un



monitoring environnemental et mis à la disposition du Musée National d'Histoire Naturel suédois à Stockholm, qui nous a donné l'opportunité d'étudier la croissance des valves de jeunes moules. Ceci nous a permis de faire des estimations d'âge très précises de la partie érodée de l'umbo des vieilles coquilles. L'abondance du matériel nous a permis d'établir une relation entre la longueur et l'âge des moules pour la Suède, de construire une courbe de croissance et par là d'aboutir à une estimation plus précise de l'âge en mesurant la longueur d'une valve. Il s'agit d'un outil performant pour les travaux de monitoring. Cependant la croissance des coquilles et par là l'âge des populations varie

en fonction des cours d'eau. Pour cela nous proposons de faire trois courbes de croissance - courbes élevées, normales et basses, pour aboutir à une relation approximative entre âge et longueur de la coquille. Bien qu'une tendance de croissance en fonction de la température ait été observée, il n'existe pas de preuve que la relation âge et longueur évolue selon un gradient biogéographique nord-sud. Il a été mis en évidence que la croissance annuelle des valves est influencée par la qualité de l'eau, telle la présence de sédiments fins. Nous démontrons que les moules perlières, surtout les stades juvéniles sont très sensibles à des changements soudains de leur environnement.

### Zusammenfassung

In den meisten Europäischen Ländern gibt es Bemühungen, die Umweltbedingungen in Fließgewässersystemen zu verbessern, um ein Überleben der sich stark im Rückgang befindenden Flussperlmuschel (*Margaritifera margaritifera*) zu ermöglichen. Um nachhaltige Erhaltungsprogramme zu entwickeln, sind wissenschaftliche Kenntnisse über alle biologischen Aspekte dieser Art erforderlich.

Im vorliegenden Artikel werden generelle Aspekte zum Schalenwachstum der Flussperlmuschel vornehmlich aus schwedischen Gewässern aber auch aus Gewässern der Kola Halbinsel dargestellt. Das Schalenmaterial, welches zum Teil auch von juvenilen Tieren stammte, wurde zwischen 1984 und 2006 von staatlichen Stellen im Rahmen von Umweltmonitoring-Programmen gesammelt und dem Naturhistorischen Museum in Stockholm zur Verfügung gestellt. Dies ermöglichte es uns sehr präzise Altersangaben auch von dem erodierten Teil des Wirbels (Umbo) zu machen. Das reichhaltige Schalenmaterial erlaubte uns den Zusammenhang zwischen der Schalengröße und dem Alter der Flussperlmuschel innerhalb der schwedischen Populationen herauszuar-

beiten und Wachstumskurven zu bestimmen. Die Ergebnisse helfen somit, dass bei Monitoring-Programmen durch Messen der Schalenlänge das präzise Alter der Tiere bestimmt werden kann. Bei der Auswertung stellte sich jedoch heraus, dass für verschiedene Fließgewässersysteme eine grosse Variabilität zwischen dem Wachstum und der Schalengröße existiert. Es wurden deshalb drei Wachstumskurven herausgearbeitet mit denen sich das Alter-Schalenlänge Verhältniss der Tiere annäherungsweise bestimmen lässt: eine normale, hohe und niedrige Wachstumskurve wurde ermittelt. Die Ergebnisse zeigen ebenfalls keinen Beleg dafür, dass es beim Alter-Schalenlänge-Verhältnis der Tiere einen biogeographischen Nord-Süd-Gradienten gibt, obwohl der jährliche Zuwachs temperaturabhängig ist. Dagegen konnte gezeigt werden, dass die jährliche Zuwachsrate von Änderungen der Gewässerqualität abhängig ist, wie zum Beispiel der Kalkung von Gewässern. Es konnte ebenfalls gezeigt werden, dass *M. Margaritifera*, und insbesondere die Jungtiere sehr empfindlich auf plötzliche Umweltveränderungen reagieren.

## Introduction

The freshwater pearl mussel (*Margaritifera margaritifera*) is widely distributed in Europe, but has suffered serious decline in the last century. Today this species is threatened by extinction and for these reasons it is listed as 'vulnerable' by the World Conservation Union IUCN and included in the Red Data List (WCMC RDL). Consequently, governmental authorities in many European countries are establishing a variety of conservation programs, in order to support the remaining endangered populations. The work to conserve this species is important not only for its biological and ecological aspects (as these mussels are

natural biological filters, indicators of good water quality and a food supply for fish and wildlife), but also for its environmental monitoring function, as their shells can record the local environmental history. The shells of these long-lived bivalves (up to 280 years) are excellent archive indicators of environmental changes, as they have solid and impermeable shells with distinct annual growth increments (similar to tree-rings) that retain incorporated elements (41 trace elements) from the ambient water without spatial relocation (Carell et al. 1987; Mutvei et al. 1994; Dunca 1999), making it possible to reconstruct palaeotemperatures and pH history (Mutvei et al. 1994; Schöne et al. 2004; Dunca et al. 2005).

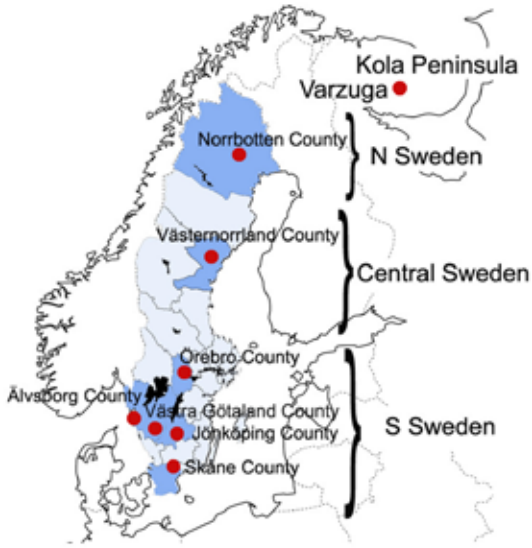


Fig. 1: Map showing the spatial distribution of the *M. margaritifera* populations in the present study.

In Sweden, along with legal protective measures, there are also major efforts to improve the environmental conditions that are necessary for the regeneration of *M. margaritifera* populations. For this purpose population profiles are periodically monitored and recruitment is estimated in all regions where these bivalves occur. Also, measures like restoring river substrates and improvement of water quality by liming, are implemented in the work of conservation.

In order to monitor the population dynamics and to evaluate the recruitment, it is necessary to estimate the age of the bivalves in relation to their shell length. This relationship is poorly studied, as the availability of shell material is restricted.

However, in the following study we have analysed the shell growth of a total of 1051 bivalves from 62 water systems. This abundant shell material was donated to the Swedish Museum of Natural

History in Stockholm by the governmental authorities in charge of environmental monitoring in seven different counties. The major aim of this study is to establish growth curves for *M. margaritifera* in Sweden, so that the age of the mussels is related to their shell length. These curves can then be employed to predict the age of each individual bivalve, if the shell length is known. Another aim is to estimate the shell growth in relation to age for a whole population by defining a growth factor,  $k$ . This growth factor can be employed in order to compare the shell growth between different mussel populations, but also to compare the shell growth to different environmental parameters, such as pH, alkalinity, or nutrient levels. The use of growth curves and growth factor could then be used to facilitate the evaluation of liming programs.

## Material and methods

In the present study 1051 bivalves from 62 streams were analysed regarding their shell growth (Fig. 1).

Most of the shells were collected live between 1984 and 2006 (Tab. 1).

Each shell was measured for length, width and height. In order to determine the age of the bivalves, thin transverse sections were made from one of the shell halves, using the same method as in previous work (Dunca 1999; Dunca et al. 2001, 2005, 2008). After being polished, the sections were coloured and etched with Mutvei's solution (Schöne et al. 2005a), which enhanced the visibility of the winter lines and in this way increased the precision of age determination (Fig. 2).

The annual increments were counted from the ventral edge to the beginning of the eroded part of each shell. In order to estimate the age of eroded parts, the age determination was carried out first with young mussels from each population. The youngest individuals (up to 10 years old) usually

Table 1: Spatial distribution, length and age range of the shell material included in our study.

| Region         | No of localities | No of shells | Age range (years) | Shell length (mm) |
|----------------|------------------|--------------|-------------------|-------------------|
| S Sweden       | 7                | 113          | 7-125             | 26-138            |
| Central Sweden | 42               | 775          | 1-190             | 3-145             |
| N Sweden       | 6                | 71           | 7-280             | 10-143            |
| Kola Peninsula | 7                | 92           | 8-149             | 13-120            |

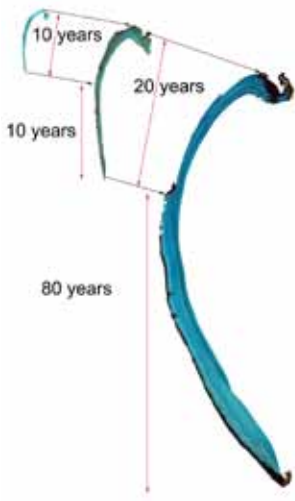


Fig. 2: Estimation of the eroded umbonal part in older shells.

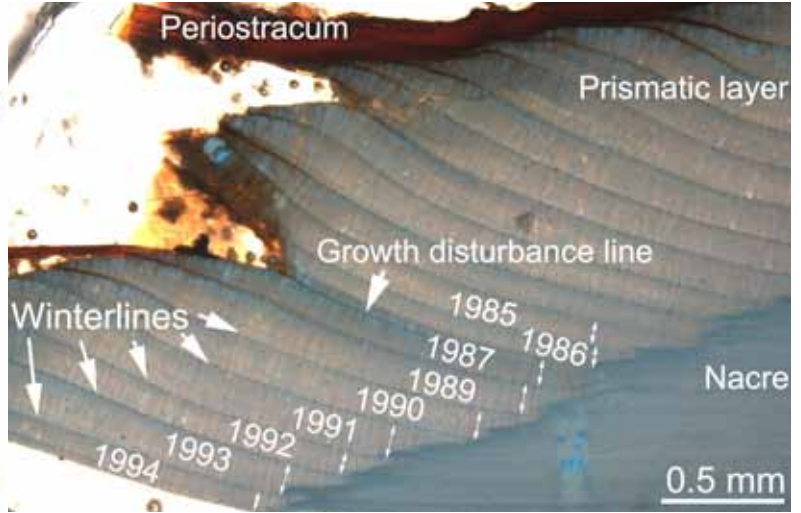


Fig. 3: Transvers thin section of a shell after treatment with Mutvei's solution, photographed in light microscope.

have an uneroded umbonal part and all annual growth increments are clearly visible. The width of these shells was then compared with the width of the eroded parts in older shells (up to ca 40 years old). In this way the age/length relationship of the eroded part was estimated from the age of the youngest mussels. Finally, the age of the oldest shells was estimated in a similar way using the previous estimations for the eroded parts (Fig. 2).

**Growth factor**

In order to compare the shell growth in relation to age between different mussel populations, and also to compare the shell growth rate with different environmental parameters, such as pH, alkalinity, nutrient levels, we calculated a growth factor, *k*, using the von Bertalanffy formula for growth (Hastie et al. 2000; Miguel et al. 2004):

$$H = L_{max} (1 - e^{-(k \cdot t)}) \quad (1)$$

where *H* = shell length, *L<sub>max</sub>* = maximal shell length, *a* = shell length year 0, *λ* = specific growth rate, *t* = time (age)

In this formula (1) there are two variables: the maximal shell length, *L<sub>max</sub>* and the specific growth rate, *λ*. In order to use the specific growth rate as a measure for general growth rate, for comparison between different populations, we have chosen to let *L<sub>max</sub>* to be a constant that represents the highest value known for the shell length of *M. margaritifera*, i.e. 170 mm (Hastie L. C. oral communi-

cation). The shell length at year 0, *a*, is ca 0.36 mm but it is not needed for the calculation of *k*. For this reason it was not taken into consideration.

Then the formula we used to calculate the growth factor, *k*, is:

$$H = 170 \cdot (1 - e^{-(k \cdot t)}) \quad (2)$$

where *H* = shell length, *k* = growth factor, *t* = time (age).

**Annual growth rate**

The annual growth rate has been examined in the mussel populations from Västernorrland County, Central Sweden. From each mussel population 2-6 individuals were chosen for growth rate analysis on an annual basis. For this purpose the transverse thin sections were photographed, using a light microscope with Carl Zeiss AxioCam camera. Each annual increment was measured as the shortest distance between two winter lines in the shell portion near the border line between the prismatic layer and the nacreous layer (Fig. 3) using Panopea image processing software (developed by Peinl & Schöne, University of Frankfurt).

Measured values exponentially decrease, as the shell growth decreases with time. This ontogenetic trend was removed, in order to be able to compare the annual shell growth between individuals at different ages. Consequently, the measurements were standardized using similar

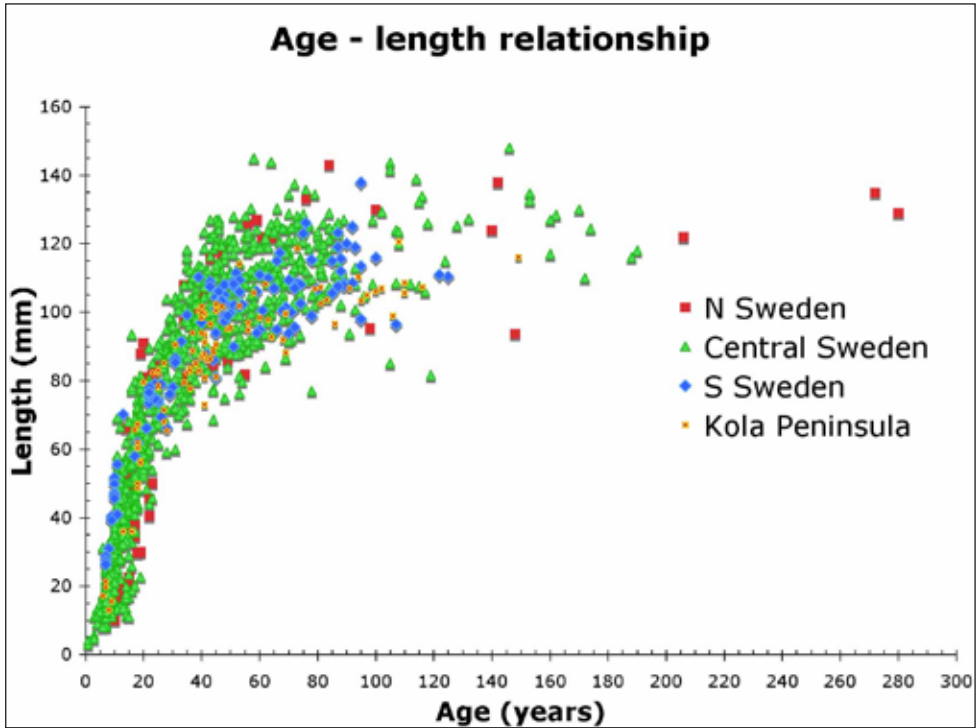


Fig. 4: Variations in the relationship between age and shell length in *M. margaritifera* populations from southern, Central and northern Sweden and from Kola Peninsula, Russia.

methods to those of dendrochronologists (Cook & Kairiukstis 1990) and described in previous work (Dunca 1999; Dunca et al. 2005, 2008; Schöne et al. 2003, 2005a, 2005b). The obtained values, i. e. the standardized growth indices (SGI), represent higher annual growth than expected if positive, or lower annual growth than expected if negative. SGI can be employed to compare the growth rate between different individuals within one population. Usually, the growth rate pattern is similar in most of the individuals belonging to the same population and a mean SGI value can be calculated for each growth season. These values estimate the annual growth rate of the population and can be compared with environmental parameters, such as annual temperature, pH, precipitation, etc. (Dunca 1999; Dunca et al. 2005, 2008; Schöne et al. 2003, 2005a, 2005b).

All the statistical analyses were carried out using the statistical tools of Microsoft Excel program.

## Results

The shell length of all 1051 mussels examined during this study ranged between 3 and 145 mm and age (growth increment counts) between 1 and 280 years.

The relationship between age and shell length varied both between and within mussel populations (Fig. 4). For example, shells that were ca 20 mm long could be from 7 to 20 years old and shells that were 110 mm long could be from 30 to 190 years old. The variation did not show any latitudinal trends, as shown in Fig. 4 where populations from southern Sweden were compared with populations from Central and northern Sweden, as well as from the Kola Peninsula.

### General growth curves

As the relationship between shell length and age varied both between and within populations it

was difficult to establish one growth curve for *M. margaritifera* in Sweden.

As a tool to help describe the relationship between age and shell length in *M. margaritifera* shells, we propose three growth curves that approximate the age - shell length relationship: the normal, high and low growth curves. For this purpose, we limited the maximal shell length for normal growth to 130 mm, for high growth to 160 mm and for low growth to 100 mm.

The curves were constructed using the von Bertalanffy formula (1). For juvenile shells up to 8 years old the growth was best fitted by exponential curves (Fig. 5).

The following formulae were used to construct these curves:

$$H_{jh} = 1 * e^{(0.433 * t)} \quad (3)$$

$$H_{jn} = 0,5 * e^{(0.438997 * t)} \quad (4)$$

$$H_{jl} = 0,2 * e^{(0.449 * t)} \quad (5)$$

$$H_h = 160 * (1 - e^{(0.05 - 0.034089 * t)}) \quad (6)$$

$$H_n = 130 * (1 - e^{(0.15 - 0.036 * t)}) \quad (7)$$

$$H_l = 100 * (1 - e^{(0.3 - 0.046923 * t)}) \quad (8)$$

where  $H_{jh}$  = shell length for juvenile mussels (0-8 years old) that had high growth rate;  $H_{jn}$  = shell length for juvenile mussels that had normal growth rate;  $H_{jl}$  = shell length for juvenile mussels that had low growth rate;  $H_h$  = shell length for mussels (>8 years) that had high growth rate;  $H_n$  = shell length for mussels (>8 years) that had normal growth rate;  $H_l$  = shell length for mussels (>8 years) that had low growth rate.

If the populations were sorted by shell growth and not by spatial distribution, then the three growth curves gave a good approximation for the growth of every population, ie each population was represented well by one of the three growth curves (Fig. 6).

These curves could then be employed to estimate the growth of mussels related to age in different populations, even if the shell samples were very few or had a narrow age distribution (Fig. 7 A).

However, in some populations younger mussels grew relatively larger than older ones with respect to their age. As example, in the River Kramforsån, Central Sweden, individuals younger than 30 years follow mostly the higher growth curve, individuals that were between 30 and 60 years old follow the normal growth curve, while individuals

that were older than 70 years followed the low growth curve (Fig. 7 B).

In the Maljan stream, Central Sweden, juvenile shells with similar age distribution, but collected at different time periods, showed a variation in shell growth as follows: juveniles collected in 1994 followed the normal growth curve and reached 42 mm in length at the age of 12 years, while the majority of the shells collected after year 2000 followed the low growth curve and only reached a length of 14 mm at the same age.

### Growth factor

The growth factor calculated for the 42 streams in Central Sweden varied between 0.01 and 0.033. There was no significant difference (student t-test,  $p > 0.05$ ) between the k values for mussel populations in limed water systems and those for populations in natural water systems.

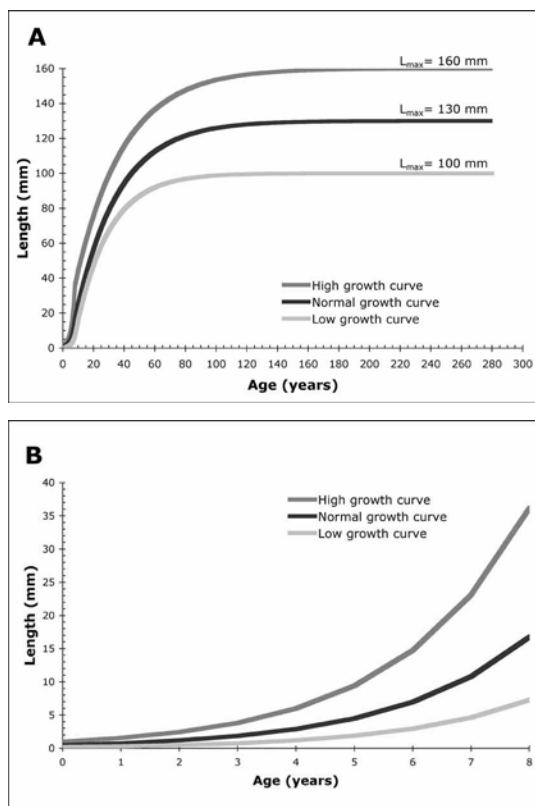
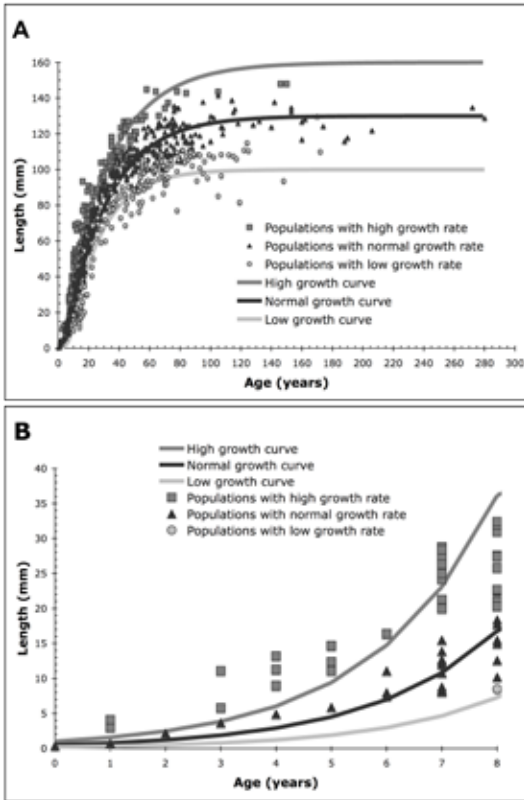


Fig. 5: A - Growth curves that approximate high, normal and low shell growth in mussels; B - Growth curves that approximate high, normal and low shell growth in juvenile mussels (younger than 8 years).





**Fig. 6:** *M. margaritifera* populations sorted by shell growth: A - all shells; B - all young shells.

Comparing the *k* values with environmental parameters, such as the mean values for pH, alkalinity, total phosphorous and conductivity, no correlations (using student *t*-test,  $p > 0.05$ ) were found for mussel populations in Västernorrland County. The only correlation found with available environmental parameters was a negative correlation between *k* values and the mean stream gradient.

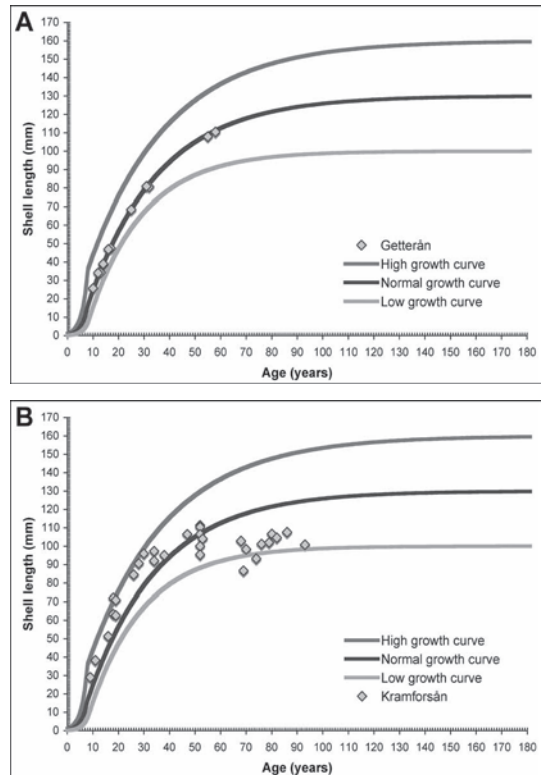
**Annual growth rate**

The analysis of the annual growth rate in mussel populations from Västernorrland County, Central Sweden, showed that there were similar trends in both limed and natural water systems (Fig. 8). However, there were significant differences (student *t*-test,  $p < 0.05$ ) in growth during the 1920s and 1930s, with mussels from limed water systems displaying higher growth rates than those in natural or unlimed river systems. There were also significant differences in growth rates during

the 1960s and 1970s, this time with mussels from limed water systems displaying lower growth rates compared with individuals living in natural or unlimed river systems. Finally, significant differences in growth rates were also observed during the 1980s, when growth rates were significantly higher in limed streams (Fig. 8, Tab. 2).

Furthermore, the mean SGI for all shells in Västernorrland County was calculated and then compared with the mean summer air temperature from this region. Trends expressed by fifth degree polynomial functions for both temperature and annual growth showed similarities (Fig. 9).

In order to evaluate the liming effect on shell growth, we put the year of liming as year zero and then averaged the SGI values for 20 years before liming and 20 years after liming in all limed

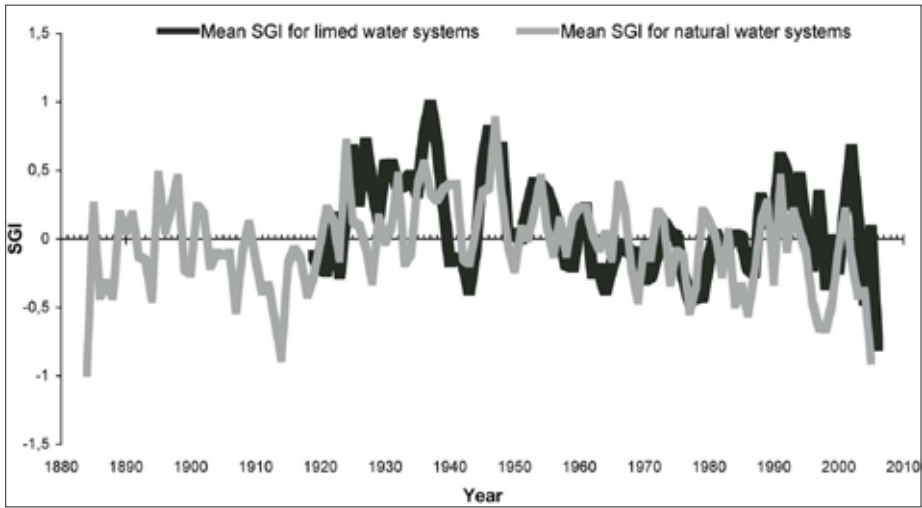


**Fig. 7:** Estimation of the shell growth in relation to age for the mussel populations in: A - Getterån River and B - Kramforsån River, Central Sweden. Ca 37% of the shells (younger than 30 years) follow the high growth curve, ca 26% of the shells (between 30 and 60 years old) follow the normal growth curve, while ca 37% of the shells (older than 70 years) follow the low growth curve.

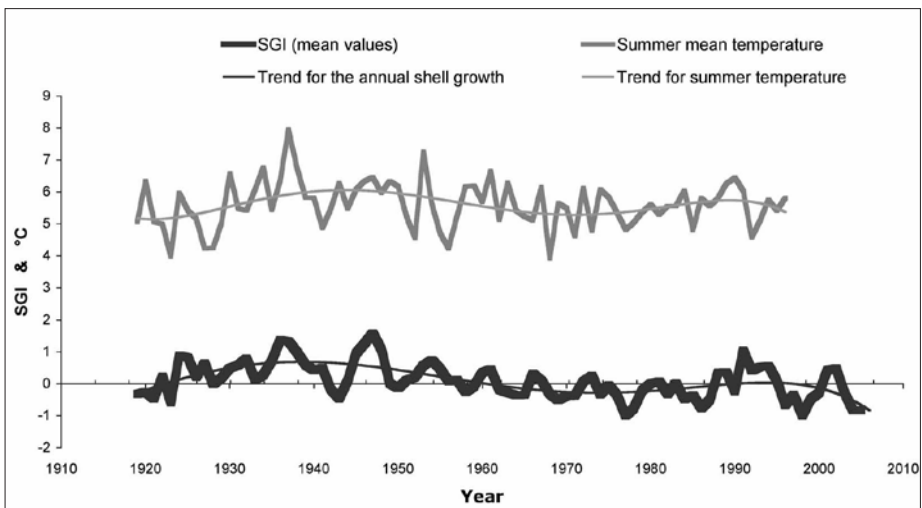


**Table 2:** Statistical analyses between the annual growth rate in shells from limed and natural streams.

| Period of time | Mean SGI |        | Variance |       | Observations | P(T<t)    |
|----------------|----------|--------|----------|-------|--------------|-----------|
|                | Natural  | Limed  | Natural  | Limed |              |           |
| 1920-1939      | 0,155    | 0,358  | 0,07     | 0,119 | 20           | *0,043    |
| 1940-1959      | 0,153    | 0,148  | 0,071    | 0,104 | 20           | *0,426    |
| 1960-1979      | -0,038   | -0,153 | 0,06     | 0,034 | 20           | *0,042    |
| 1980-2005      | -0,193   | 0,073  | 0,11     | 0,08  | 25           | ***0,0009 |



**Fig. 8:** Comparison between annual shell growth in limed and natural water systems.



**Fig. 9:** Comparison between annual shell growth and summer mean temperatures. Trends were calculated using a fifth degree polynomial function.

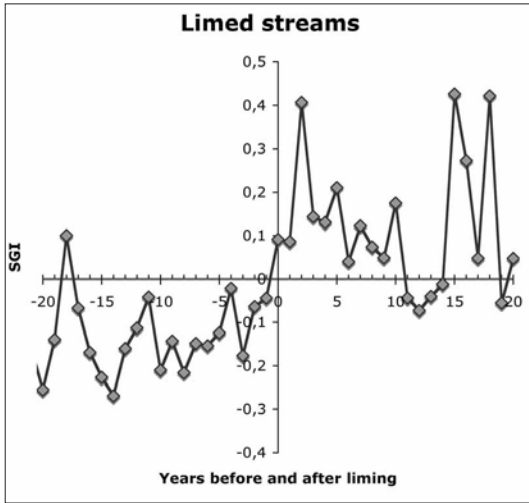


Fig. 10: Liming year as year zero shows that 20 years after liming the SGI values are higher than for the 20 years before liming.

streams. This was necessary as the liming started at different time periods (between 1981 and 1993) in most of the streams. Considering year of liming as year zero also eliminated the influence of temperature on shell growth. The SGI values (i. e. annual growth) after liming were higher than before liming (Fig. 10).

While measuring the annual increments many growth disturbances were observed in most of the analysed shells. In some populations these growth disturbances occurred more often than in others. In order to evaluate the growth disturbance occurrence in a population, the annual increments with growth disturbance lines were counted and expressed as % of the total number of annual increments measured in a population.

Populations from limed water systems were compared with those from natural streams with respect to the frequency of growth disturbances, but no significant difference was found (student t-test,  $p > 0.05$ ).

## Discussion

In the work of monitoring population dynamics, it important to estimate as accurately as possible the age of the mussels by measuring their shell length. Our aim in the present project was to establish the

relationship between age and shell length for the freshwater pearl mussel, *M. margaritifera*, and to offer a useful tool for the practical work of monitoring the population dynamics. However, because there is substantial variability in the age-shell length relationship, both between and within populations from the same region, it is not possible to construct a single growth curve model that accurately predicts the age of a shell by measuring its length. We propose therefore the use of three growth curves that describe normal growth, high growth and low growth. These calibration curves can then provide relatively reliable age estimations. In order to use these curves it is necessary to analyse several shells in order to establish which curve is appropriate for the respective population and for this purpose shells from dead mussels are useable. Then it is possible to use the most closely matched of the three generalised growth curves (for normal, high and low growth) in order to estimate the age of other mussels in the population.

However, the most reliable estimation can be achieved by constructing a specific growth curve for each population. Unfortunately, this procedure requires shell material from at least 15 mussels at different ages. It is important to know that the growth curves can be constructed even using dead collected shells, as long as they have the carbonate part preserved.

Most of the freshwater pearl mussels in Sweden have a normal growth in relation to their age. However, there are populations in which young mussels grow larger than usual for their age, but not the older ones, and consequentially the mussels in the population do not follow only one growth line. One possible explanation is that in these streams the water quality may have changed in time due to liming, acidification or fertilization. An example is the Kramforsån River that was limed from 1986. Younger shells may have responded to liming with increased growth rate but not the mature shells that were already old (over 40 years old) at the time of liming. Another explanation could be that the shell growth is restricted by genetic inheritance and shell growth is inhibited at a certain age. Further studies are needed to clarify this problem.

Recent investigations on shells from Majlan, Västernorrland County, Sweden, have shown that fertilization near water system (and near the mussel population) in combination with liming of

the source lake affect the growth rate of juvenile mussels. All juvenile shells show a growth disturbance line in the annual increment for 1989, the year of the first liming. It has been estimated that 70 % of the juvenile mussels had lower growth rate in the past 20 years (Dunca et al. 2009). Majlan stream is included in a Natura 2000 area and it is relatively unaffected by human activity other than liming and fertilization of the forest. The mussel population in this stream is one of the most viable populations in Sweden, having the best recruitment rate (Eriksson et al. 1998). It is not yet clear what our observations show. The variations in pH of the water (due to both liming and fertilization) may negatively affect the shell growth, causing many growth disturbances that would affect mostly the juveniles. It may be the opposite: more juvenile mussels may survive and even those mussels that in more normal conditions would perish may then have the possibility to mature, due to the better environmental conditions brought on by liming and fertilisation (neutral pH and better food supply). It is not clear either how liming and fertilization will influence the mussel populations in a longer time perspective.

Our observation that liming increases the annual growth rate in shells gives rise to the question: is there a positive influence on the mussel population if the growth rate increases? Does the shell growth reach a normal growth level (that the mussels would reach if the water system had an optimal pH) or does the growth rate increase above normal due to liming and does this have negative effects, for example, on breeding. Our results show also that liming in the region of Västernorrland does not increase the % of growth disturbance, although it is known that high dose liming can produce growth disturbances (Mutvei et al. 1996). This may show that the liming program in this region uses optimal, rather than excessive, doses. However, further investigations are necessary in order to evaluate the effects of liming and fertilization on long term population dynamics and shell growth of freshwater bivalves.

The growth factor,  $k$ , can be used to explore how differences in the regional environment influence shell growth. The value of  $k$  can be compared with different environmental factors as pH, conductivity, alkalinity, etc.. No significant correlation was found for  $k$  values for the populations in Västernorrland County and environmental parameters

other than with the stream slope. The correlation is negative, which suggests that in streams with higher stream slope, i. e. higher water velocity, there is usually a lower shell growth in mussels. The other environmental parameters in this region are within the optimal range (for example, pH that varies between 6.4 and 7.5), and so do not influence the shell growth.

In the present study we also compared the overall  $k$  values of populations from limed streams with the ones from natural streams. No significant difference was found for Västernorrland region. A possible explanation is that the liming occurred only in the last 20 years and the generation after liming is not old enough to influence the  $k$  value. Furthermore, in older mussels the shell growth is less representative, as the length increases less than 0.5 mm per year and the effect of liming does not show clearly in shell length measurements. An option is to calculate the  $k$  value separately for mussel generations after liming. Another explanation could be that the measurements of the environmental parameters that were available for our study were not made on a periodic basis and are not as representative as mean values. Future studies involving other geographic regions within Sweden, and better data sets on environmental parameters, would be necessary in order to elucidate how liming influences the growth factor,  $k$ .

There is no evidence that the age - shell size relationship of *M. margaritifera* has a North - South biogeographical gradient. The growth rate varies among the southern populations, as well as among the northern populations, in a similar way.

The natural variations in the summer temperature and the variation in annual growth rate of all shells studied from Västernorrland County show similar trends. This is in agreement with results from previous studies on other mussel populations (Dunca & Mutvei, 2001, Dunca et al. 2005, Schöne et al. 2004). This indicates that the main regulatory growth factor is temperature, both in limed and natural streams.

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# Developing strategies for introductions of captive-bred *Margaritifera margaritifera* (L.) into the wild

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## Abstract

A key method of species conservation is captive breeding and release into the wild. This is usually the option of last resort when the organism of concern is either locally or functionally extinct. The endangered freshwater pearl mussel *M. margaritifera* has suffered huge declines across its biogeographic range, and as a result there is much interest in captive breeding and release as a conservation tool to halt its decline. This paper outlines an experimental protocol, which attempts to adhere to IUCN guidelines for reintroductions as far as is possible. The objective is to augment a declining *M. margaritifera* population in the Ballinderry River, Northern Ireland using juvenile mussels bred from captive stock from the natal river. Four hundred and ninety five mussels grown in captivity since 1998 have been tagged using Passive Integrated Transponder (PIT) tags. Fifteen tagged mussels were used in a pilot study, five of which were a control group to

monitor stress during transport. Nine of the remaining ten have been relocated using the PIT tag receiver, and five of these have been visually confirmed as alive; none of the mussels in the control group suffered any ill effects as a result of transport. From the remaining tagged mussels, approximately 350 individuals in two size classes will be released across three sites within the river. Two periods of release were carried out; February and September 2009. Mussels were randomly allocated across three sites and taken to each site to be released, along with a control group to test for the effect of stress during transport on mussel mortality. Growth, survival and dispersal of translocated mussels will be monitored at intervals of six months. In this study, we provide an example of a scientifically informed protocol for introductions of captive-bred *Margaritifera margaritifera* (L.) into the wild to supplement declining populations or re-establish the species where it has become extinct.

## Résumé

Dans la conservation d'une espèce, l'élevage en captivité et le relâchement constituent une méthode clé. Il s'agit d'habitude de l'option du dernier recours quand l'organisme en question est localement éteint ou manque de recrutement. La moule perlière *Margaritifera margaritifera* a souffert d'un important déclin dans toute son aire biogéographique. C'est pour cette raison que l'élevage en captivité et le relâchement sont d'un grand intérêt pour la conservation de l'espèce et pour freiner le déclin. Cette publication résume un protocole expérimental qui tente de s'aligner autant que possible aux directives de l'IUCN pour la réintroduction d'une espèce. L'objectif en

est d'augmenter l'effectif d'une population de *Margaritifera margaritifera* en déclin dans le cours d'eau Ballinderry en Irlande du Nord en utilisant des jeunes moules perlières élevées à partir d'un stock du cours d'eau natal. Depuis l'année 1998 les 495 moules qui ont grandi en captivité ont été marquées avec des émetteurs passifs (Passive Integrated Transponder PIT). De ces moules marquées 15 ont été utilisées dans une étude dont 5 ont servies de référence pour surveiller le stress pendant le transport. Neuf des dix restantes ont été relocalisées en utilisant le PIT receveur et 5 ont été confirmées vivantes de manière visuelle. Aucune des 9 moules du groupe



de référence n'a souffert du transport. Des moules marquées restantes environ 350 sont classées en fonction de leur taille en 2 groupes et seront réparties à 3 endroits dans le cours d'eau. Le relâchement des moules sera réalisé à 2 périodes: une fois au mois de février et une deuxième fois au mois de septembre 2009. Les moules étaient attribuées au hasard aux 3 sites et y seront transportées, chaque fois avec un groupe de référence pour

contrôler l'impact du stress du transport sur la mortalité des moules. La croissance, la dispersion et la survie des moules déplacées seront contrôlées tous les 6 mois. Dans cette étude nous fournissons un exemple d'un protocole scientifique pour le relâchement de *Margaritifera margaritifera* élevées en captivité et pour soutenir ainsi les populations en déclin ou pour réintroduire l'espèce où elle est éteinte.

### Zusammenfassung

Eine Schlüsselmethode im Artenschutz ist die Nachzucht in Gefangenschaft und das spätere Freilassen in die Natur. Dies ist eine der letzten Methoden wenn die Art bereits lokal verschwunden ist oder ein Überleben in der Wildnis ohne Unterstützung nicht mehr möglich ist. Die stark bedrohte Flussperlmuschel *Margaritifera margaritifera* verzeichnet in ihrem biogeographischen Einzugsgebiet sehr starke Populationsrückgänge. Aus diesem Grund gibt es viele Bemühungen, die Art nach zu züchten und durch Auswildern vor dem Aussterben zu bewahren. Dieser Artikel stellt ein experimentelles Protokoll vor, welches sich weitestgehend an die IUCN Richtlinien für die Wiedereinbürgerung von Tieren hält. Das Ziel ist es, die abnehmende Population von Flussperlmuscheln im Ballinderry Fluss in Nordirland durch Besatz zu unterstützen. Hierfür wurden juvenile Muscheln gezüchtet, die Nachkommen von Tieren aus dem Ursprungsfluss sind. 495 Muscheln die seit 1998 in Gefangenschaft groß gezogen wurden, wurden mit PIT Transpondern (Passive Integrated Transponder) markiert. 15 Muscheln wurden im Rahmen einer Pilotstudie markiert, fünf davon waren Kontrollmuscheln,

um die Stressfaktoren während des Transports zu untersuchen. Neun der verbleibenden zehn Tiere konnten mit Hilfe eines PIT-Empfängers wieder gefunden werden und fünf konnten visuell als lebendig identifiziert werden. Keine der fünf Kontrollmuscheln zeigte irgendwelche Schwächesymptome, die auf den Transport zurück zu führen waren. Von den übrig gebliebenen markierten Muscheln wurden ungefähr 350 Individuen in zwei verschiedenen Größenklassen an drei verschiedenen Stellen im Fluss ausgesetzt. Die Auswilderung wurde zu zwei verschiedenen Zeitpunkten durchgeführt: Februar und September 2009. An den drei Probestellen wurden Muscheln zufällig gesammelt und wieder freigelassen, zusammen mit einer Kontrollgruppe, um die Sterblichkeit während des Transportes zu testen. Größe, Überlebensrate und Verteilung der lokalisierten Muscheln wurden über sechs Monate in Intervallen aufgezeichnet. In dieser Studie zeigen wir das Beispiel eines wissenschaftlichen Protokolls für die Auswilderung von *Margaritifera margaritifera* (L.) um verschwindende Populationen aufzustocken oder um Spezies dort wieder einzuführen, wo sie verschwunden sind.

## Introduction

Species conservation may involve controlling exploitation, habitat protection or restoration, translocation or captive breeding and release (Fitter 1986), or a combination of these approaches. The deliberate movement of animals within their range is known as **translocation** and is considered a **reintroduction** when attempting to re-establish a species within its historic range where it has become extinct or extirpated. Animals used for reintroduction may be taken from the wild at another location or generated in captive-breeding programmes. If a population is small or declining in an area it may be augmented by **supplementation**, also using either captive-bred or wild animals from another location (IUCN 1998).

Captive breeding and release has been primarily used in conservation of mammals and birds

(Fischer & Lindenmayer 2000) and has not been without controversy (Alonga 2004). In a review by Fischer & Lindenmayer (2000) only 7% of 181 translocation studies, including releasing captive-bred animals into the wild, were of amphibians, reptiles and invertebrates, which implies that a disproportionate amount of resources are allocated to bird and mammal conservation. Successful examples of invertebrate captive breeding and reintroduction include the Karner Blue Butterfly (*Lycaeides melissa*) (Tolson et al. 1999) in Ontario, Canada, the Apollo Butterfly (*Parnassius apollo*) in the Pieniny National Park (Polish Carpathians) (Witkowski et al. 1997), and the American Burying Beetle (*Nicrophorus americanus*) (Perrotti et al. 2001). However, captive breeding and release should be deemed a last resort and not a long-term solution to species' decline (Snyder et al. 1996; see also Geist 2005).



Captive breeding and release programmes should include genetic analyses of individuals used as brood stock, individuals to be released and recipient wild populations (IUCN 1998; Geist & Kuehn 2005; Jones et al. 2006; Hoftzyer et al. 2008). Other information gathered should include the status and biology of wild populations, a detailed knowledge of the habitat needs of the species of concern (IUCN 1998; Dunn et al. 1999) and suitable release sites with adequate long-term protection (IUCN 1998). Before releasing captive stock it is important to determine the reasons for the decline and whether they have been removed or sufficiently reduced (IUCN 1998; Jones et al. 2006). Post-release activities should include long-term direct or indirect monitoring of individuals or a subsample (Kurth et al. 2007). This ensures objective assessment of the success of captive breeding and release as a conservation strategy for the species of concern (IUCN 1998; Jones et al. 2006). In the 1990s, a number of authors emphasised the need for scientific monitoring of re-introductions (Armstrong et al. 1994; Sarrazin & Barbault 1996; Seddon 1999). However, research in this area is still ad hoc, fragmented and in need of more structured studies (Armstrong & Seddon 2007; Seddon et al. 2007).

*Margaritifera margaritifera* is a long-lived freshwater bivalve that historically occurred in high densities in oligotrophic rivers and streams throughout Europe and the Eastern seaboard of North America (Skinner et al. 2003). When it occurs in dense aggregations it acts as a keystone species by filtering river water and providing habitat, food and refuge for invertebrates (Vaughn & Hakenkamp 2001). *M. margaritifera* has a complex life cycle involving a parasitic stage on a salmonid host. Declining host fish, poor water quality, river engineering, overfishing and siltation have resulted in huge declines of the species across most of its biogeographic range (Bauer 1983; Bauer 1988; Young 2001). Consequently the species is listed as Endangered A1ce+2c on the IUCN Red Data List (IUCN 2008) and is protected in Europe under Annex 2 and Annex 5 of the Habitats Directive 92/43/EEC. This is achieved by designating *M. margaritifera* sites as Special Areas of Conservation (SACs), whereby the species and their habitat are protected. Under the Northern Ireland Species Action Plan (SAP) for *M. margaritifera* key targets include halting the decline and maintaining the



Fig. 1: A PIT tagged captive-bred juvenile *M. margaritifera*.

range of current populations, increasing the size of protected populations by 50% by 2010 and re-establishing populations in two former suitable localities by 2020 (Anon 2005). It is also recognised in the European SAP that there is a need to improve mussel habitat and water quality in *M. margaritifera* catchments (Araujo & Ramos 2001; see also Geist 2005). Despite these various methods, many populations of pearl mussels are over-aged and continue to decline. In areas where populations are functionally extinct it may be necessary for them to be augmented using mussels from another location or from captive-breeding programmes to meet EU directive(s). Translocation as a conservation tool for *M. margaritifera* has been carried out with mixed success. Restocking *M. margaritifera* populations by trans-

plantation has been almost twice as successful when carried out within a river as opposed to transplanting between rivers (Valovirta 1995a cited in Valovirta 1998), potentially emphasising the need for more research on genetic analyses of wild populations. Captive breeding of *M. margaritifera* is increasingly being used throughout Europe as a conservation tool (Buddensiek 1995; Hastie & Young 2003; Preston et al. 2007; McIvor & Aldridge 2008). Translocation is not an option in Northern Ireland because there are no populations of pearl mussels large enough to be used for such purposes.

This study, which is licensed by the Northern Ireland Environment Agency, outlines the rationale and strategy for initiating experimentally controlled trials to supplement the Ballinderry River *M. margaritifera* population using captive-bred animals and following, where possible, the IUCN guidelines. It aims to develop scientifically informed protocols for introductions of captive-bred *M. margaritifera* (L.) into the wild to supplement declining populations or re-establish the species where it has become extinct.

## Materials and methods

The Ballinderry River has a small and dispersed population of around 800-1000 adult *M. margaritifera* (Killeen 2007). As a result of a successful captive breeding programme using brood stock from the Ballinderry River (Preston et al. 2007) there are now over 700 juveniles with ages estimated to range from four to ten years old. Thus, we are now in the unique position of being able to address the ultimate aim of captive breeding, i.e. to introduce these animals into the river where their parents originated. In addition, the genetic diversity of the brood stock used for captive breeding in Northern Ireland is highly representative of the wild stock (Wilson et al. unpublished data). This meets the first of the IUCN guidelines listed above. The status of wild populations of *M. margaritifera* in Northern Ireland, including those in the Ballinderry catchment, is such that intervention is necessary at this time.

Although not fully understood there is a broad recognition of the key physical habitat require-

ments of freshwater pearl mussels (see e.g. Hastie et al. 2000). Habitat was deemed suitable when the banks were tree-lined and the substrate contained a good mix of clean gravel stabilized with cobble/boulder. On the basis of this information three sites have been identified for trial introductions of captive-bred *M. margaritifera*.

Passive Integrated Transponders (PIT tags) have been used with some success to monitor Eastern Lampmussels (*Lampsilis radiata radiata*) where it was shown they improve mussel recapture from 30-47% using visual searches only, to 72-80% when using PIT tags and visual confirmation (Kurth et al. 2007). PIT tags are glass-encapsulated microchips that lie inert until interrogated by an inductive coil PIT tag receiver. The receiver (ANT 610f-IP68) displays a unique 12 digit alphanumeric code displayed on an LCD (LID 650 decoder) when the tag is located. Mussels cultivated in Northern Ireland, between the ages of four and ten years old were tagged using 12mm PIT tags (Trovan® ID100 Unique) (MID Fingerprint Ltd., UK; Dorset Identification, Aalten, Netherlands). In preparation for tagging, the mussels were taken and dried thoroughly using paper towels; the surface of the shell was cleaned using 100% ethanol and then lightly sanded to roughen the surface of the shell; which helps the epoxy resin to bond with the shell. Once the surface of the shell had completely dried, a small amount of epoxy resin was applied, on top of which a PIT tag was set. Each PIT tag ID along with the mussel length was recorded. The mussels were left exposed to the air with some water covering the lower valve to keep them cool until the epoxy set fully. Mussels could be left in this condition for up to thirty minutes before being returned to their tank. Initially, fifteen mussels were tagged in this way, returned to their tank and revisited two weeks later to check for any adverse effects. All these mussels survived and tagging of the remaining mussels was resumed. After tagging, mussels (n = 495) were maintained in cultivation tanks until release.

In the hatchery the mussels were sorted into two size classes: 40 - 45.99 mm (small) and 46 - 50 mm (large). Mussels were released using the following protocol. Individuals were chosen from each size class and randomly assigned to one of three release sites. A total of 28 individuals were randomly selected from the small size class and 33 from the large, per site. Each time a group of

mussels was taken to the release site, a control group was also taken consisting of five randomly selected mussels from each size class. Each individual of the control group was treated in the same way as the experimental animals. These mussels will be used to determine whether the stress of transport is a factor in mussel mortality after release. Once the mussels reached the release site, they were gently pushed into the substrate after making a small hole with a dibber. A detailed record of individual mussels in each site and their location will be maintained. Three sites are being used over the river, and will be treated as replicates as they all have similar habitat. Survival will be monitored on a monthly basis and growth will be monitored every six months. At each sampling interval mussels will be relocated using the PIT tag receiver.

To test the behaviour of the mussels when released, a pilot study was carried out. Fifteen PIT tagged mussels were removed from captivity at the Ballinderry Fish Hatchery, their identity was recorded using the PIT tag receiver and they were taken to the release site. A site of suitable habitat (as described above) was selected and ten mussels were randomly chosen, gently pushed into the substrate and their location accurately mapped. The substrate consisted of cobbles and clean coarse gravel. The remaining five individuals were used as a control group to assess the impact of transport on them and taken back to the hatchery.

## Results

None of the individuals in the control group in the hatchery have suffered any ill effects as a result of the translocation process. Two months after release, nine of the ten released mussels in the pilot study group were relocated; seven were confirmed as alive because they can be seen in the sediment. Fourteen months after release two mussels were found in the release plot still alive, the remainder were either burrowed too deep or had been washed out due to severe flooding events that occurred in August and December 2009.

Trials began in February 2009 with the release of 84 large and 65 small juveniles; a second similar release was carried out in September 2009. Mussels were transported to the release sites along

the Ballinderry River (2-6 km away) in buckets containing river water. However, it is too early as of yet to determine the efficacy of this study as it is a long term commitment.

## Discussion

Post-release monitoring is often inadequate in reintroduction studies (Seddon et al. 2007). This problem may be overcome by using PIT tags, which ensures more efficient recovery of experimental mussels (Kurth et al. 2007). The preliminary results of the present study have demonstrated that the process of attaching the PIT tags to the mussels using Epoxy resin has no effect on their survival. Other release studies carry out random quadrat sampling to determine survival success (D. Neves personal communication, 2008); however these studies involve much larger scale releases that rule out the use of PIT tags due to cost. Due to the burrowing nature of *M. margaritifera*, difficulties can be found in recapturing them if they are deep in the sediment or if the individual is small. The read range of the PIT tag receiver is dependent on the size of PIT tags used. In the present study the read range is around 10 – 15 cm, so the receiver's search coil is brushed over the river sediment when searching for the mussels. As the juvenile mussels are small, 12mm long PIT tags were used. There are larger PIT tags available that will increase the read range, but it is recommended that these be used for adult mussels. If burrowed in the sediment, it can also be difficult to determine whether the individual is alive without gently moving the sediment away, this can be a time consuming process. However, this process is still much less invasive than without PIT tags

The potential for mortality as a result of transport to the release sites will be determined through the use of a control group. This is an important aspect to consider (Hartup et al. 2005; Teixeira et al. 2007) because translocation can potentially result in high mortalities (Teixeira et al. 2007). Initial results from a pilot study show that the translocation of juvenile mussels using the method described is not detrimental to survival. However, it is important to consider the use of a control group when transporting mussels from hatchery to release sites, as it will help identify the cause of any mortality. Not all mussel translocations will be the same;

time of year, expertise of staff involved and the distance the mussels are transported will vary and have different impacts on mussel survival. In addition, Dunn et al. (1999) emphasize the importance of avoiding temperature extremes during translocation, and identify studies where this may be a cause of mortality. This may be done by carefully timing the mussel translocation when air and water temperatures are closest, i.e. summer in Northern latitudes (Dunn et al. 1999). Two release phases are planned, the first took place in February 2009 and the second phase will begin in summer 2009.

Translocation studies which involve hypothesis testing will ultimately further our knowledge of the effectiveness of captive breeding and release for *M. margaritifera* conservation, as well as contribute to the broader field of reintroduction biology (Armstrong et al. 1994; Sarrazin & Barbault 1996; Seddon 1999). In the proposed study described above several questions will be addressed: At what age should mussels be reintroduced back into the wild? At what time of year should mussels be released? How far do they move after release? Are PIT tags as effective when used on smaller mussels? Does the translocation process have any adverse effects on mussel survival? These are all important questions that will ultimately advance our understanding of the benefits and disadvantages of releasing captive-bred pearl mussels to augment the recovery and conservation of declining populations of freshwater mussels.

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