Department für Ökosystem- und Landschaftsmanagement

Fachgebiet Wildbiologie und Wildtiermanagement

Conservation Genetics and Ecology of European Freshwater Pearl Mussels (*Margaritifera margaritifera* L.)

Jürgen Geist

Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan

für Ernährung, Landnutzung und Umwelt der Technischen Universität München

zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften (Dr. rer. nat.)

genehmigten Dissertation.

Vorsitzender:	Univ.-Prof. Dr. R. Schopf
Prüfer der Dissertation:	1. Univ.-Prof. Dr. W. Schröder
	2. Univ.-Prof. Dr. A. Melzer
	3. Ao.Univ.-Prof. Dr. R. A. Patzner, Universität Salzburg / Österreich

Die Dissertation wurde am 22.03.2005 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 17.05.2005 angenommen.

The freshwater pearl mussel (*Margaritifera margaritifera* L.)

Science is facts; just as houses are made of stones,

so is science made of facts;

but a pile of stones is not a house

and a collection of facts is not necessarily science.

Henri Poincare, French mathematician and physicist (1854-1912)

Contents

List of figures vi

List of tables viii

Preface ix

1 Introduction 1
  1.1 Statement of problem 1
  1.2 Objectives 4

2 The freshwater pearl mussel 6
  2.1 Systematics and phylogeny 6
  2.2 Distribution and populations 7
  2.3 Life history 11

3 Development of microsatellite markers for the endangered freshwater pearl mussel *Margaritifera margaritifera* L. (Bivalvia: Unionoidea) 14
  3.1 Abstract 14
  3.2 Introduction 14
  3.3 Material and Methods 15
  3.4 Results and Discussion 16

4 Genetic diversity and differentiation of central European freshwater pearl mussel (*Margaritifera margaritifera* L.) populations: implications for conservation and management 19
  4.1 Abstract 19
  4.2 Introduction 20
  4.3 Materials and Methods 21
    Sampling strategy 21
    DNA isolation and microsatellite analyses 25
    Statistical and population genetic analyses 25

4.4 Results ... 27
Linkage and Hardy-Weinberg equilibrium ... 27
Genetic diversity and relatedness within populations ... 28
Genetic differentiation between populations ... 31
4.5 Discussion ... 37
Population structure ... 37
Conservation and management implications ... 40
4.6 Conclusions ... 42

5 The potential of using mollusc shells for DNA-based molecular analyses ... 44
5.1 Scope of using mollusc shells for DNA-analyses ... 44
5.2 Introduction ... 44
5.3 Applications of shell DNA analyses ... 45
5.4 Factors influencing the success of shell DNA analyses ... 46
5.5 Recommendations ... 48

6 Stable carbon isotopes in freshwater mussel shells: Environmental record or marker for metabolic activity? ... 50
6.1 Abstract ... 50
6.2 Introduction ... 51
6.3 Material and Methods ... 53
Sampling site ... 53
Shell preparation ... 55
Stable carbon isotope analysis ... 56
Statistical analysis ... 57
6.4 Results and Discussion ... 57
Sampling method and reliability ... 57
Mussels display long-term $\delta^{13}C$ patterns ... 60
Carbon sources for aragonite formation ... 63
Mussels show individual signals ... 66
6.5 Conclusions ... 70

7 The status of host fish populations and fish species richness in European freshwater pearl mussel (*Margaritifera margaritifera*) streams 72
7.1 Abstract 72
7.2 Introduction 73
7.3 Material and Methods 75
Sampling area 75
Electrofishing and data analyses 79
7.4 Results 81
Species richness and fish biomass 81
Host fish biomass and host fish density 84
7.5 Discussion 87
The status of host fish populations 87
Implications for management and conservation 91

8 General Discussion 93
8.1 Aspects of conservation on the individual and population level 93
8.2 Aspects of conservation on the species level 97
8.3 Aspects of conservation of global biodiversity 100
8.4 Recommendations for future research 102

9 Summary 104

10 Zusammenfassung 106

11 References 108

List of figures

Fig. 1.1: Freshwater pearl mussels (*Margaritifera margaritifera* L.) of different age classes .. 2

Fig. 2.2: Length-frequency distributions of two pearl mussel populations............................. 11

Fig. 2.3: Life cycle of the freshwater pearl mussel (*Margaritifera margaritifera* L.) 12

Fig. 4.1: Sampling locations (black circles) of freshwater pearl mussel (*Margaritifera margaritifera*) populations in central Europe and magnification of the sampling sites at the contact zone between the three main drainage systems of Elbe, Main/Rhine and Danube 24

Fig. 4.2: Heterozygosity contribution *CT* to total diversity (subdivided into a diversity and a differentiation compound) for 24 central European freshwater pearl mussel (*Margaritifera margaritifera*) populations... 31

Fig. 4.3: Neighbour-Joining (NJ) phenogram based on $NeiD_A$ (Nei *et al.* 1983) genetic distance for central European freshwater pearl mussel populations 32

Fig. 5.1: Freshwater pearl mussel (*Margaritifera margaritifera*) shells of different age and degradation (A, B, C) and electrophoresis of PCR-products of a species specific STR locus (D) with template DNA from theses shells... 45

Fig. 5.2: Factors influencing quantity and quality of DNA and subsequent molecular analyses and their impacts on errors during data-analyses 47

Fig. 6.1: Schematic of the cross-section of a *Margaritifera margaritifera* shell 52

Fig. 6.2: Instantaneous measurements of pH and temperature from the brook water between 1987 and 2003 54

Fig. 6.3: Number of years per sample of the outer and inner layers as estimated from visual inspection 58

Fig. 6.4: Correlation of the $\delta^{13}C$ signature of shell carbonate from the last (1998-2000) outer layers with the youngest (2001) shell carbonate (A) and with the organic carbon in body tissue (B) 61

Fig. 6.5: Pooled semivariograms of $\delta^{13}C$ signature for the inner, outer and all layers 62

Fig. 6.6: Comparison of shell signature (crosses for outer layers; open symbols for inner layers) with atmospheric CO_2 signature (filled symbols) reconstructed from annual averages from the Siple icecore (Antarctica; Friedli *et al.*, 1986), and the atmospheric measuring stations of Mauna Loa (Hawaii, Keeling *et al.*, 1995), Hungary, Ulan Uul (Mongolia) and Ochsenkopf... 64

Fig. 6.7: Variation of $\delta^{13}C$ between successive prismatic layers of both valves from mussel Z3 67

Fig. 6.8: Comparison of $\delta^{13}C$ patterns from all four mussels... 69

Fig. 7.1 Electrofishing sites (black circles) in European freshwater pearl mussel streams 76

Fig. 7.2: Comparison of *Salmo trutta* biomass and densities between streams with and without trout stocking (A) and between rivers with and without reproduction of freshwater pearl mussels (B) 86

Fig. 7.3: Correlations of species richness with host fish biomass (A) and species richness with host fish density (B) in European freshwater pearl mussel streams........................ 87

List of tables

Tab. 2.1 Revised estimates of current population status of *M. margaritifera* in Europe.......... 9

Tab. 3.1: Characteristics of 13 microsatellite loci for the freshwater pearl mussel (*Margaritifera margaritifera*) 18

Tab. 4.1: Samples used for genetic analyses 23

Tab. 4.2: Microsatellite diversity indices for central European freshwater pearl mussel (*Margaritifera margaritifera*) populations..... 30

Tab. 4.3: Pairwise estimates of F_{ST} between central European freshwater pearl mussel (*Margaritifera margaritifera*) populations..... 35

Tab. 4.4: Assignment test for freshwater pearl mussel (*Margaritifera margaritifera*) based on the Bayesian Method..... 36

Tab. 5.1: Authenticity criteria to determine shell DNA-data..... 48

Tab. 6.1: Parameters of spherical models fitted to the experimental semivariograms of the outer and inner layers 62

Tab. 6.2: Average C content, C to N ratio and C and N signatures of potential food sources..... 64

Tab. 6.3: Average $\delta^{13}C$ and $\delta^{15}N$ signatures of mussel tissues 65

Tab. 7.1: Characterisation of electrofishing sites; Drainages, Subdrainages, Populations, Codes and Countries..... 77

Tab. 7.2: Characterisation of fish species distribution in 20 European freshwater pearl mussel rivers..... 83

Preface

This work is intended to contribute to conservation strategies for freshwater pearl mussels in particular and aquatic molluscs in general by demonstrating that sustainable conservation strategies can benefit from a holistic and interdisciplinary research approach, integrating both molecular genetic and ecological studies.

The thesis is structured as follows: An introduction describing the scope and the goals of this study (chapter 1) is followed by a chapter providing essential background information about systematics and phylogeny, distribution and current population status and the life history of pearl mussels (chapter 2). Chapters 3-7 address five specific research topics in the context of freshwater pearl mussel conservation genetics and ecology, each of them representing an autonomous research paper (published, in press or submitted in a slightly modified format, according to the journal requirements). These specific aspects merge into a general discussion about conservation strategies (chapter 8).

Originally, this interdisciplinary PhD project on “Conservation Genetics and Ecology of European Freshwater Pearl Mussels (*Margaritifera margaritifera* L.)” was intended to include a small selection of pearl mussel populations from Bavaria plus a few additional reference samples from other areas. During the course of the project, a network has evolved, comprising about 80 scientists of 15 countries. During field expeditions, sampling trips and other research cooperation, many of the working relationships with my colleagues developed into very good friendships. I am particularly grateful to the following people who all contributed physically and mentally to the success of this work:

At the Technische Universität München – Center of Life Science Weihenstephan most of the work was conducted under supervision of Dr Ralph Kühn (Principal Supervisor and Head of the Molecular Ecology and Conservation Genetics Group), Prof. Dr Wolfgang Schröder (Wildlife Biology and Wildlife Management Unit), Prof. Dr O. Rottmann (Animal Biotechnology Group), Prof. Dr H. Stein †(Fish Biology Unit) and Prof. Dr K. Auerswald (Chair of Grassland Science). I especially appreciated the intense support to freely follow my scientific ideas, the excellent working conditions, the stimulating discussions and the friendly atmosphere at their institutes.

I am also grateful to Prof. Dr H. Schnyder (Chair of Grassland Science), Prof. Dr Kögel-Knabner (Chair of Soil Science), Dr H.H. Becher (Chair of Soil Science) and Dr R. Schäufele (Chair of Grassland Science) for supporting this project by providing access to additional laboratory resources for stable isotope and sediment analyses, and to Dr M. Holzner (Fish Biology Unit) for providing electro-fishing generators. C. Gerschlager is acknowledged for taking care about the administration affairs linked with the project. I am particularly grateful to my colleagues, to my co-workers/HIWIs and M.Sc. students A. Beck, C. Bottlender, C. Scheuring, B. Reindl, K. Schullehner, H. Wunderlich, and to all interns who joined my pearl mussel research team and who helped with electrofishings during the course of the project.

I am grateful to “Landesfischereiverband Bayern e.V.” and “Bayerischer Naturschutzfonds” for funding this project. Dr O. Born, Dr S. Hanfland (Landesfischereiverband Bayern), B. Schäffler and Dr H. Kleisinger (Bayerisches Landesamt für Umweltschutz) were involved with the project plans from the beginning and their contributions, e.g. during the selection of Bavarian pearl mussel streams for the investigations, are acknowledged. Most investigations would not have been possible without special licences, e.g. according to species protection laws, animal treatment laws, natural protected areas laws, fishing legislation, and pearl fishing legislation. In most cases the required licences were kindly provided in time for each of the investigated populations by different government authorities. Additionally, local forest services, private landowners and people holding private fishing rights are acknowledged for giving their consent to investigations in particular streams.

I am particularly grateful to the following people for their support in Germany: Dr R. Klupp (Fischereifachberatung Oberfranken), Dr T. Ring (Fischereifachberatung Oberpfalz), Dr W. Silkenat and Dr P. Wondrak (Fischereifachberatung Unterfranken), Dr Harsányi and J. Hoch (Fischereifachberatung Niederbayern), Dr R. Altmüller and R. Dettmer (Niedersächsisches Landesamt für Umweltschutz), Dr P. Pongratz (Bezirksregierung Oberfranken), Dr H. Stetter (Bezirksregierung Oberpfalz), Ch. Schmidt, G. Wenz and Dr R. Vandré (Schmidt&Wenz GbR, LIFE 2002NAT/D/8458), Dr K.-O. Nagel and W. Fricke (Pearl mussel project Vogelsberg), St. Schmidt, G. Nowak, E. Späth and the staff of Wasserwirtschaftsamt Hof, F. Elender (Landschaftspflegeverband Passau e.V.), M. Lange (Interreg IIIa EEV 2138) and the “Anglerverband Südsachsen e.V.”. I especially acknowledge the great help of my parents, Franz and Christine Geist, who often had to be my field

assistants for investigations in Bavarian pearl mussel streams during week-ends and at times when no one else was available, despite the fact that neither of them have any particular interest either in mussels or in biology.

During the course of this project, I was able to visit and investigate a series of pearl mussel populations abroad and discussed various aspects of research and conservation with my colleagues and friends from the "pearl mussel community", which intensively widened my point of view on the topic. Without the hospitability of the following people these investigations and the intense scientific exchange would not have been possible:
G. Meier-Lehner, Dr Th. Ofenböck, Mag. J. Moser (Austria), Dr E. Moorkens (Ireland), I. Killeen (England), Dr M. Young (Scotland), K. Greke and D. Telnov (Latvia), Dr R. Gross and N. Laneetu (Estonia), E. Holder, Bretagne Vivante and the "Fédération de pêche du Finistère" (France), St. Terren, Dr G. Motte, St. Bocca (Belgium, LIFENature B/02/8590), K. Groh, G. Weitmann (Luxembourg/Germany), J. Hruška, D. Jäger, G. Ritter, M. Formánková (the Czech Republic), M. Porkka, K. Autio, S. Kankaanpää and the staff of Urho Kekkonen National Park (Finland). Dr T. von Proschwitz, L. Henriksson (Sweden), R. Araujo (Spain) and J. Reis (Portugal) provided additional samples. Dr D. L. Dettman, T. McConnaughey (USA) and Dr A. Boom (United Kingdom) are acknowledged for their contributions to stable isotope analyses.

Dr E. Moorkens helped whenever I had specific questions concerning the correct use of English language and M. Porkka spent a lot of time discussing specific pearl mussel research issues together with me. I also thank a series of anonymous reviewers from various journals, in which parts of this work were published or accepted.

Last but not least, I am grateful to the head of the PhD examination commission, Prof. Dr R. Schopf and to the jury committee members Prof. Dr W. Schröder, Prof. Dr A. Melzer and Prof. Dr R. Patzner for their contributions.

1 Introduction

1.1 Statement of problem

Molluscs are an extremely diverse group of animals with more living species than birds, mammals, reptiles, amphibians and fishes combined (Lydeard & Lindberg, 2003).
Thus, they are an important segment of the overall biodiversity. Many of the molluscs have important functions in ecosystems.

The global decline of nonmarine molluscs is causing increasing concern (Lydeard *et al.*, 2004). In particular, freshwater bivalve molluscs have shown severe declines during the last decade with many species now facing extinction. Freshwater mussels are probably the most endangered groups of animals (e.g. Bogan, 1993, 1998; Williams *et al.*, 1993; Neves *et al.*, 1997; Strayer *et al.*, 2004). Given the high biomass and the high original abundances (c. hundreds of mussels per square metre) and thus the important roles of bivalve molluscs in particle processing, nutrient release, and sediment mixing (for review see Vaughn & Hakenkamp, 2001), the decline of originally dense mussel populations can have manifold implications on the functioning of aquatic ecosystems. Despite their importance, there is often a lack of knowledge about their complex biology, which connects the processes that influence their rapid declines.

One example is the freshwater pearl mussel (*Margaritifera margaritifera* L.), a long-lived highly threatened bivalve occuring in cool running waters of the Holarctic region. Some authors even consider it to be one of the most endangered freshwater mussels in the world (Machordom *et al.*, 2003). About one century ago, freshwater pearl mussels still occurred in high densities, often covering the stream bottom in several layers (Israel, 1913). It has been estimated that there has been a decline of more than 90% in European populations up to the 1990s (Bauer, 1988), a trend that has obviously continued or even increased. The current main concern is the lack of juvenile reproduction in most European pearl mussel populations.

Direct threats for adult mussels like pearl harvesting, predation by muskrats and eel (potentially feeding on juvenile mussels) have limited local influence and cannot explain the species´ global decline. Instead, indirect effects connected with anthropogenic perturbations such as habitat degradation, alteration and fragmentation are probably the most important

factors for decline. A lack or decline of host fish populations and a series of additional interferences with the chemistry, biology, hydrology and geomorphology of streams may also have contributed to the current imperilment of pearl mussels.

Most European pearl mussel populations have lacked successful reproduction for 30-50 years and in many cases their original distribution has been dramatically reduced. Thus, formerly dense and connected populations have often become fragmented and reproductively isolated remnant and island populations. However, a great potential for recovery is offered by the longevity of this species, i.e. a lifespan of more than 100 years (Bauer, 1992), together with the high reproductive potential that adult pearl mussels show, even in polluted rivers and in extreme old age.

Fig. 1.1: Freshwater pearl mussels (*Margaritifera margaritifera* L.) of different age classes

Early conservation efforts have most often focussed on the effects of abiotic habitat factors on species (autecology) and on the complex relationships between species (synecology) with the intention of giving detailed descriptions of the species´ habitat requirements. Conservation planning has tended to focus more on pattern (representation) than process (persistence) and,

for the former, has emphasized species and ecosystem or community diversity over genetic diversity (Moritz, 2002).

More recent conservation approaches have shown that ecological studies can greatly benefit from a combination with genetic studies. Genetic investigations into the extent and organisation of genetic diversity in populations and its spatio-temporal dynamics are a powerful tool to suggest sustainable conservation strategies. In particular, small and isolated populations can suffer from the effects of genetic drift and the loss of genetic variability, which contribute to inbreeding and rapid extinctions of such populations (extinction vortex). On the other hand, thorough ecological investigations are needed in order to reveal the specific requirements that must be fulfilled in the habitat during all life stages of the species. Both ecological and genetic reasons alone can lead to extinctions of populations, and understanding of the interaction of ecological and genetic factors may determine the dynamics, local occurrence or extinction of mussels. New research disciplines of Conservation Ecology and Conservation Genetics address these questions. The conservation of biodiversity between and within species have become priority goals, thus retaining the evolutionary potential for adaptation to future changes in the environment.

As with other freshwater bivalves, an integrative conservation approach that identifies and sustains ecological processes and evolutionary lineages is urgently needed to protect and manage freshwater pearl mussel diversity. Such research is important for the conservation of free-living populations, as well as for artificial culturing and breeding techniques, which have recently been or which are currently being established for freshwater pearl mussels in several countries.

1.2 Objectives

Overall Objective

The overall objective of this study is to contribute to the conservation of freshwater pearl mussels, choosing an integrative approach of combining conservation genetics and ecological investigations in order to deduce conservation strategies. As several different deterministic and stochastic factors linked with the species´ genetics and ecology can all contribute to the phenomenon of decline with different intensities in different regions, investigations addressing different hypotheses and objectives were carried out in different geographical regions.

There were six specific conservation genetics objectives and five specific ecological objectives, as follows:

Conservation Genetics Objectives

- To develop high resolution polymorphic microsatellite markers for *Margaritifera margaritifera* which allow monitoring of neutral genetic diversity and differentiation in order to describe the current population genetic structure of pearl mussel populations
- To establish a non-destructive sampling method of DNA from living mussels (haemolymph) which is harmless and suitable for endangered bivalves, and to discuss the potential use of shell DNA for genetic analyses
- To assess the genetic structure within the last remaining central European pearl mussel populations (e.g. inbreeding coefficients, heterozygosities, history of populations, bottlenecks and founder effects, influence of life history and population history on genetic structure, effects of genetic stochasticity on small populations)
- To assess genetic parameters among populations within drainages (e.g. genetic diversity and differentiation, demographic influences on the genetic structure of closely related populations, gene flow versus drift, spatial differences of genetic parameters within drainages)
- To assess genetic parameters between populations over different drainages (e.g. overall population structure, differentiation and diversity of connected and isolated populations, selection of priority populations for conservation and definition of

Conservation Units, CUs; assignment of population origin and detection of past stocking activities from other populations)

- To recommend conservation strategies for free-living populations, supportive breeding and culturing techniques on a genetic basis

Ecological Objectives

- To assess the status of host fish populations in pearl mussel populations and to compare fish communities and the fish species richness in pearl mussel streams with and without recruitment of juvenile pearl mussels
- To assess and discuss the influence of supportive conservation measures such as trout stocking on host fish densities and biomass for specific populations
- To develop a method of sampling annual growth layers from the outer prismatic and inner nacreous zone of pearl mussel shells and to assess their applicability as reliable long-term environmental archives
- To assess the influence of environmental and metabolic variables on shell carbonate stable isotope $\delta^{13}C$ signatures within a time-scale
- To detect the origin of carbon in mussels shell aragonite, to assess the influence of different potential carbon sources on shell aragonite formation and to assess the trophic level and origin of food for pearl mussels with stable isotope $\delta^{15}N$ studies

2 The freshwater pearl mussel

2.1 Systematics and phylogeny

Freshwater mussels and clams are members of the class Bivalvia within the genus Mollusca. The large freshwater bivalves belong to the order Unionoida (= naiads, Unionacea) and had evolved from an as yet unidentified marine group by at least the Triassic (Watters, 2001). Bivalves of the order Unionoida are a diverse group of freshwater organisms (about 175 genera) with a broad distribution that currently includes all continents except Antarctica (Haas, 1969a; Roe & Hoeh, 2003). The Unionoida nominally include two superfamilies, the Etherioidea and Unionoidea, distinguished by larval forms (Parodiz & Bonetto, 1963; Haas, 1969b; Heard & Gluckert, 1970; Davis & Fuller, 1981; Boss, 1982). The Etherioidea (Muteloidea), with lasidia larvae, includes the Etheriidae (Africa, South America) and Iridinidae (Africa). The Unionoidea, with glochidia larvae, include the Hyriidae (Australasia, South America), the Unionidae (Africa, Eurasia, India, North America) and the family Margaritiferidae (Eurasia, North America), which are considered to be a basal and primitive clade within the Unionoidea (Haas, 1969a; Smith & Wall, 1985; Smith, 2001).

In his revised classification of the Margaritiferidae based on conchological, anatomical, biological and ecological characters, Smith (2001) proposes 12 margaritiferid species and suggests a classification into the three genera *Pseudunio* (five species), *Margaritinopsis* (six species) and *Margaritifera*, with *Margaritifera margaritifera* being the only species of the genus. Recent investigations into the phylogenetic relationships of the Margaritiferidae based on molecular data, however, indicate that the group is in need of revision since the genus is not monophyletic and the taxonomy by Smith (2001) is not supported (Huff *et al.*, 2004). Hypotheses on the historical geographical dispersal of the Margaritiferidae conflict. Some authors assume that early dates of wide clade distribution suggest the break-up of the supercontinent Pangea as the cause for dispersal (Smith, 2001; Davis & Fuller, 1981), but it is alternatively suggested that colonisation might have occurred more recently when salmonid hosts released juvenile margaritiferids onto the North American continent (Marchordom *et al.*, 2003).

In Europe, two extant species of pearl mussels are described, *M.* (*Pseudunio*) *auricularia* (Spengler, 1793), an almost extinct species occurring in Southern Europe, and *M. margaritifera* (L., 1758), both of which encompass a number of contentious or uncertain taxa of lesser rank. Especially the taxonomic status of the last remaining population of the critically endangered Irish hardwater species/subspecies *M. (m.) durrovensis* (Phillips, 1928) has been a matter of several scientific discussions (e.g. Chesney *et al*., 1993; Moorkens & Costello, 1994; Chesney & Oliver, 1998). Recent investigations support that it is an ecophenotype of *M. margaritifera* (Machordom *et al*., 2003). It is often stated that the systematics of European naiads have been a battlefield for very different opinions with few other groups having been subject to so many controversies on the number of species involved, their distinction and their phylogenetic relationships (Nagel *et al*., 1998). With *M. margaritifera* a number of disputed and uncertain taxa of subspecies rank have arisen due to the wide range of shell shapes and textures observed between populations (Chesney & Oliver, 1998), demonstrating the need for thorough genetic investigations instead of an over-reliance on highly variable morphological shell characters.

2.2 Distribution and populations

The freshwater pearl mussel (*Margaritifera margaritifera* L.) is a Holarctic species, being distributed from the arctic and temperate regions of western Russia, westwards through Europe to the northeastern seaboard of North America (Jungbluth *et al*., 1985). With only a few exceptions, pearl mussels are exclusively found in rivers and streams which are extremely low in lime and nutrients.

The most accurate and detailed reviews of the current distribution and population status of freshwater pearl mussels are available from Sachteleben *et al*. (2004), Young *et al*. (2001a) and Araujo & Ramos (2000). However, all of them lack some information due to recent rediscoveries, declines and extinctions of some populations. Figure 2.1 and Table 2.1 attempt to provide information on the current distribution and populations of pearl mussels considering the most accurate data available, based upon recent publications, a series of personal communications in the year 2005, and personal survey work carried out during the course of this project in the years 2003 and 2004. It has to be noted, however, that no reliable information is available for certain geographical regions due to a lack of recent survey work, as indicated in Figure 2.1 and Table 2.1.

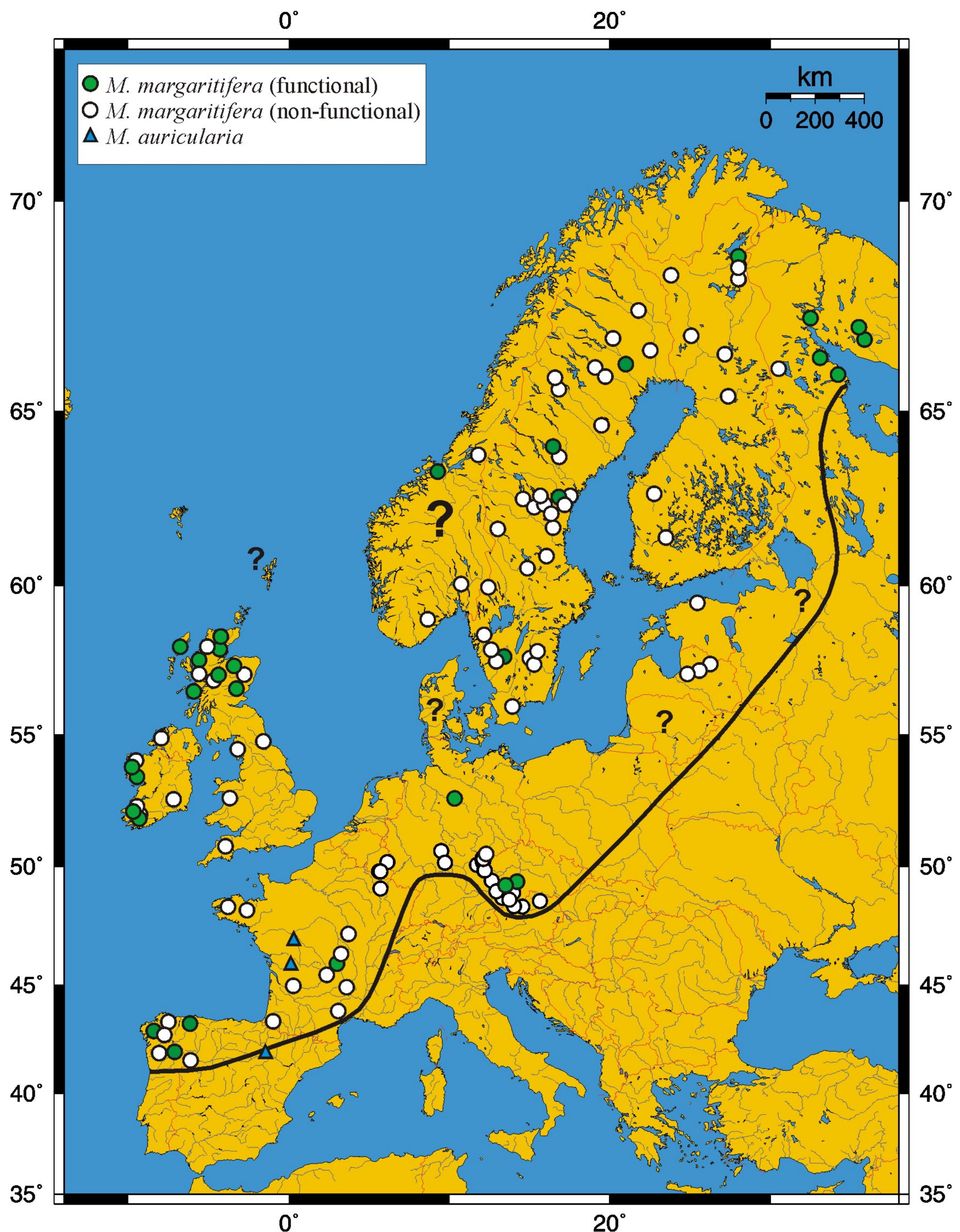


Fig. 2.1: Pearl mussel distribution and populations in Europe. Green circles indicate secure current *M. margaritifera* populations with significant percentage (>20%) of juveniles younger than 20 years; white circles indicate secure populations from recent surveys without proof of sufficient juvenile recruitment; the blue triangles represent the probably last remaining *M. auricularia* populations in Europe. The black line refers to the southern distribution limit of *M. margaritifera* in Europe. Note that single spots can refer to population units comprising more than one population and that the actual numbers of populations remain unclear for some geographical regions, indicated by question marks.

Country	Estimated number of populations	Estimated number of individuals	Current status
Austria	29	50,000	Only three large populations; strong decline; less than 5 populations with limited juvenile recruitment
Belgium	5-6	2,500-3,000	Almost extinct populations with lack of juvenile recruitment; conservation programmes since 2002
Czech Republic	6	80,000	3 populations at frontier streams plus 3 populations with more than 20% of juveniles but only one of them being large (60,000 individuals); first European country with a culturing station for pearl mussels (established by J. Hruška)
Denmark	max. 1	0?	Probably extinct, last record from 1970
Estonia	1	35,000-40,000	Lack of juvenile recruitment for at least 40 years
Finland	50	1,500,000	Largest remaining population in Lutto drainage, Northern Finland; 75% of populations lost in 20th century; 11 important populations remain; some populations with few juveniles, but probably only few functional populations
France	84	max. 100,000	Scarce in most of former range; originally abundant in more than 200 rivers; at present less than 10 rivers with juveniles; populations still present in Massif Amoricain (18), Massif Central (57), Morvan (6), Vosges (1) and Pyrenees (2) but serious declines; one big population in Dronne (16,000 individuals) with little recruitment; other populations mostly small with 10 to 100 individuals, max. 300 individuals
Germany	69	max. 144,000	Still present, largest populations with > 10,000 individuals in Bavaria but serious declines; only one recovering population with >20% juveniles in Northern Germany; several conservation and breeding programmes have started
Great Britain	>105	>12,000,000	Best populations in Scotland but 2/3 of the originally known 155 populations extinct; overall still >12,000,000 mussels with one river estimated at 10,000,000 alone. 10 rivers with significant numbers of juveniles and common or abundant adults, 5 others with some juveniles but scarce adults; England: 10 pearl mussel rivers remain (the best population has >100,000 mussels but few juveniles and evidence of declining); Wales: 10 rivers (the best has <1,000 mussels)
Ireland	110	10,000,000	Best rivers between 2 and 3 mio. individuals, most in the hundreds or a few thousand; serious decline with few recruiting populations; 90% of Northern Ireland populations lost; *M. (m.) durrovensis* almost extinct
Latvia	8	25,000	Serious decline, no population with juvenile recruitment remains
Lithuania	1?	?	Status unknown
Luxembourg	1	1,000-1,500	Almost extinct; conservation programme planned
Norway	340-350	Probably millions	Serious decline, especially in the South; exact distribution, total numbers and juvenile status unclear
Poland	0	0	Extinct
Portugal	6	>1,000,000	Severe decline, 3 large populations (22,000; 50,000; 1 million) with evidence for juvenile recruitment remain, but serious declines expected in two of them due to recent construction of man-made dams
Russia	>8	>100,000,000	Serious decline, four populations of over 1 million remain, probably representing the best European populations; good recruitment in certain areas
Spain	19	?	Serious decline; at least 17 populations in Galicia, 1 in Asturias and 1 in Salamanca; probably no more than 2 reproductive populations with significant numbers of juveniles
Sweden	>400	>8,000,000	Serious declines, but in at least 50 populations with "significant" numbers of juveniles <50mm

Tab. 2.1 Revised estimates of current population status of *M. margaritifera* in Europe. Information partly based on data and references in Sachteleben *et al.* (2004), Young *et al.* (2001a), Araujo & Ramos (2000), Alvarez-Claudio *et al.* (2000), Velasco Marcos *et al.* (2002), Morales *et al.* (2004), Larsen (2001), Rudzite (2004), Reis (2003), and updated information according to personal communications with M. Porkka, C. Greke, M. Rudzite, D. Telnov, St. Terren, G. Motte, J. Reis, E. Moorkens, I. Killeen, M. Young, G. Cochet, F. Renard-Laval, E. Holder, P. Durlet, T. Ofenböck, J. Hruška, N. Laanetu, L. Henrikson, T. von Proschwitz, E. San Miguel Salán, R. Araujo, and from personal survey work.

In Europe, the species was originally widespread and formed the basis for significant pearl fisheries. At present, the largest European pearl mussel populations with several million individuals and an intact age structure occur in Russian rivers of the Kola peninsula (Ziuganov *et al*., 2001). Big populations are also reported from Scandinavia and the British Isles, with Scotland still holding a large number of important populations (Young & Williams, 1983). Pearl mussel distribution in the south of the species´ range on the Iberian peninsula was originally considered to be limited to a few small populations in Northern Spain (Bauer, 1986), until important and reproductively active populations have recently been rediscovered in Portugal (Reis, 2003).

The largest central European pearl mussel populations are found in the drainages of the Elbe, the Danube, the Weser, the Main/Rhine and the Maas, comprising the countries of Germany, the Czech Republic, Austria, Belgium and Luxembourg. Additionally, a number of (usually small) populations still exist in France (Massiv Central, Arquitaine, Brittany) and in the Baltic States. Significant numbers and proportions of juveniles that justify a classifaction of the populations as sustainably "functional" only occur in a handful of European populations in the countries of Germany (Lutter), the Czech Republic (Blanice), Portugal (Douro tributaries), Scotland (several rivers), Ireland (Western populations), Northern Scandinavia (e.g. Pikku-Luiro) and Russia (e.g.Varzuga drainage). A number of additional populations show limited reproduction which will probably not be enough to secure the current status of these populations. The vast majority of European populations are extremely overaged, with the youngest individuals usually being 30-50 years old and with no juvenile mussels detectable during intensive surveys (Figure 2.2).

The global decline of freshwater pearl mussel populations in the last 50 years has attracted much concern from national and international conservation organisations (Araujo & Ramos, 2000; Strayer *et al*., 2004). They are currently listed in the European Habitats & Species Directive Annexes II and V, the Bern Convention Annex 3, and are a priority species in many European Biodiversity Action plans.

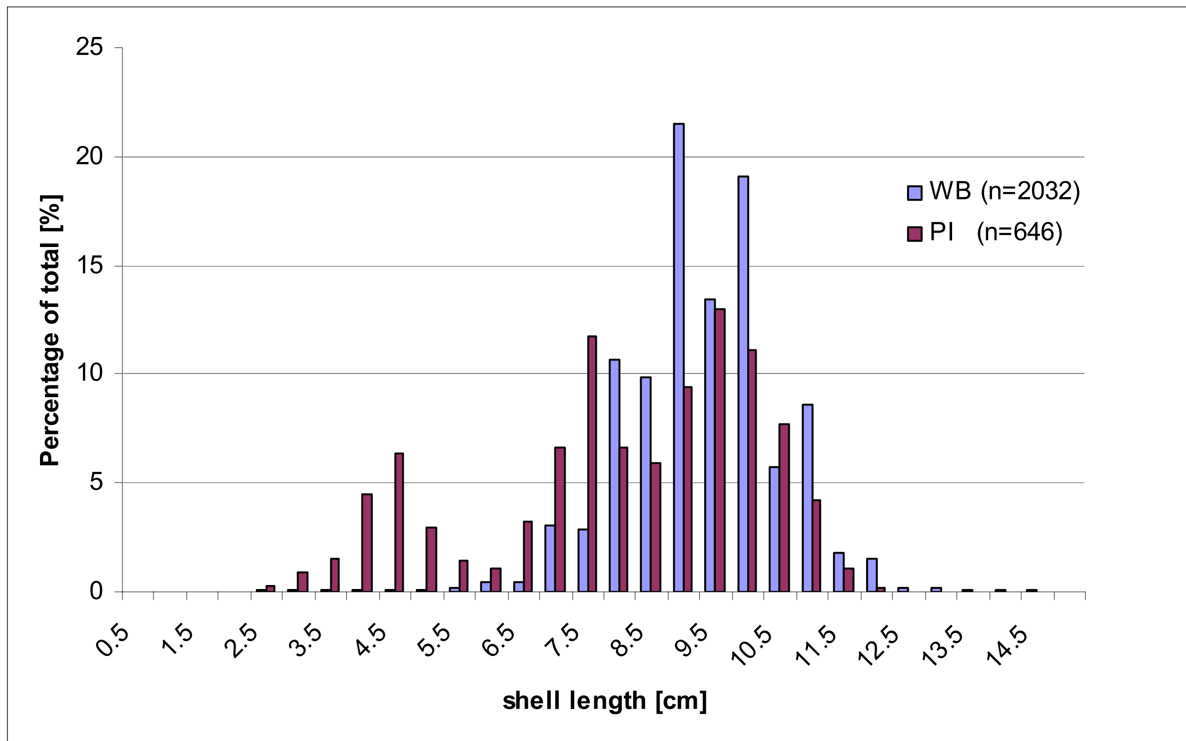


Fig. 2.2: Length-frequency distributions of two pearl mussel populations, one of them considered to be functional (PI, Northern Lapland), and one overaged population (WB, central Europe) showing a distinct lack of juvenile reproduction. Mussels <2.5 cm cannot be reliably counted in field surveys. Note that pearl mussels show asymptotic growth and that interruptions of juvenile recruitment even happened in the functional population.

2.3 Life history

Like all other large freshwater mussels of the order Unionoida, *Margaritifera margaritifera* is characterized by a semi-infaunal mode of life in its adult phase, being partly buried into the substrate. Adult pearl mussels can actively move by pumping haemolymph into their foot, but they are very sessile in comparison with other naiads.

Freshwater pearl mussels are among the longest-lived invertebrates known, frequently reaching ages of more than 100 years (Bauer, 1992) and a maximum length of 15 cm. The maximum age reached is highly variable between populations and seems to primarily depend upon growth rates. Populations tend to be faster growing and shorter lived in the southern part of their range with Spanish populations only attaining 35 years (Miguel *et al.*, 2004), whereas pearl mussels in cooler Scandinavian climates can reach ages of up to 200 years (Mutvei & Westermark, 2001) or more (Geist & Porkka, in prep.).

As with all unionoid mussels, freshwater pearl mussels have a complex life-cycle (Figure 2.3). In common with other freshwater bivalves, the sexes of *M. margaritifera* are usually separate but females were observed to become hermaphrodites at low population densities (Bauer, 1987a). The complex reproductive strategy of freshwater pearl mussels is marked by a high fertility resulting in a single female producing several million larvae (glochidia) per year (Young & Williams, 1984). In mid- to late summer the glochidia are discharged into the river. A recent study estimated daily peak releases up to 441 million glochidia per day for a Scottish population (Hastie & Young, 2003b). The proportion of adults producing glochidia is relatively high even in sparse populations (Young & Williams, 1983; Hastie & Young, 2003b; Schmidt & Wenz, 2000; Schmidt & Wenz, 2001), and reduced fecundity does not seem to be the limiting factor preventing juvenile recruitment in most pearl mussel populations.

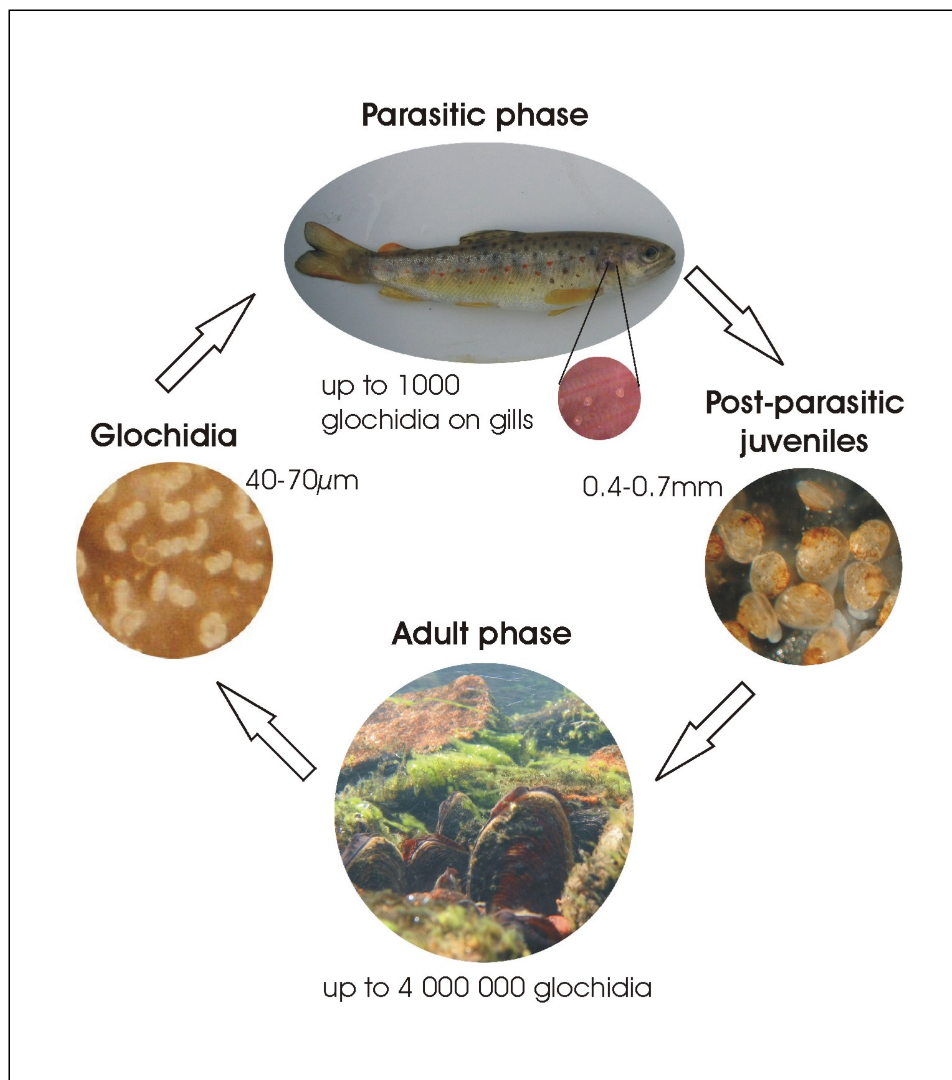


Fig. 2.3: Life cycle of the freshwater pearl mussel (*Margaritifera margaritifera* L.)

Viable freshwater pearl mussel populations are highly dependent on viable host fish populations. In the first stage of the life-cycle after their release, the glochidia of *M. margaritifera* must be inhaled by a suitable host fish, where they live encysted as obligate gill-parasites for a period of up to 10 months (Bauer, 1994). Glochidia only remain infective for a few days and over short distances downstream of the sites from where they are released (Jansen *et al*., 2001). Only sea trout (*Salmo trutta* f. *trutta*), brown trout (*Salmo trutta* f. *fario*) and Atlantic salmon (*Salmo salar*) are known to host complete metamorphosis in Europe, where they are the only native host species (Young & Williams, 1984). Salmon appear to be the main hosts in Nova Scotia (Cunjak & McGladdery, 1991) and Russia (Ziuganov *et al*., 1994). In central Europe, brown trout are reported to be the preferred or the only available hosts (Bauer 1987b, c; Wächtler *et al*., 2001).
Glochidial rejection is not only limited to non-host fish. Many fish hosts become progressively resistant to glochidial infection (Young & Williams, 1984; Bauer & Vogel, 1987; Ziuganov *et al*., 1994).

It remains uncertain if pearl mussels can be considered to be parasites only, as their host fish may benefit from the reduced suspended organic material in river water by filter-feeding by the mussels. Additionally, mussel beds can also provide important microhabitats for juvenile salmonids and the aquatic invertebrates upon which they feed (Hastie & Cosgrove, 2001). Ziuganov & Nezlin (1988) consider the relationship between mussel and fish to be a variety of symbiosis-protocooperation.

During their post-parasitical phase, juvenile pearl mussels bury themselves into the stream sediments for a period of five years, where they depend upon a stable substrate with high sediment quality (Buddensiek *et al*., 1993; Geist, 1999a, b).
The huge losses involved in this extraordinary life-cycle make the freshwater pearl mussel particularly vulnerable to adverse conditions (Skinner *et al*., 2003).

3 Development of microsatellite markers for the endangered freshwater pearl mussel *Margaritifera margaritifera* L. (Bivalvia: Unionoidea)

published: Jürgen Geist, Oswald Rottmann, Wolfgang Schröder and Ralph Kühn (2003): Development of microsatellite markers for the endangered freshwater pearl mussel *Margaritifera margaritifera* L. (Bivalvia: Unionoidea); *Molecular Ecology Notes* **3**, 444-446

3.1 Abstract

Freshwater pearl mussels (*Margaritifera margaritifera* L.) are among the most critically endangered freshwater invertebrates. We describe the isolation and characterization of the first microsatellite markers for this species, which were obtained by screening 4,900 recombinant clones from two genomic libraries. Thirteen loci revealed polymorphisms as demonstrated on 42 tested individuals from four river drainages. Allelic richness ranged from two to twelve alleles and averaged 6.8 alleles per locus with heterozygosity levels varying from 0 to 0.850 for observed heterozygosity (H_o) and from 0.174 to 0.838 for expected heterozygosity (H_e). Deficiency of heterozygous genotypes was observed in eight of thirteen loci.

3.2 Introduction

The freshwater pearl mussel (*Margaritifera margaritifera* L.) is considered to be among the most endangered freshwater mussels in the world (Machordom *et al*., 2003). Therefore great importance is attributed to its conservation throughout central Europe. Within its geographical range, the species originally occurred in high population densities. During the last century, most populations have declined by more than 90%, in turn affecting the complex mating system. At low densities, females can switch to hermaphrodites with dominating self-fertilization (Bauer, 1987a), presumably resulting in lower genetic diversity and fitness of the offspring. In most European populations reproduction has not been successful for more than 30 years despite the fact that sufficient gravidity has been observed. Therefore, conservation efforts focus on semi-artificial breeding and culturing techniques for juvenile mussels as a conservation tool. Both the poor knowledge about genetic effects and the extraordinary reproduction strategy of the species require an effective analytical tool such as highly variable

microsatellites to examine current levels of genetic variability within and between populations as a basis for culturing and protection. Our initial attempts to transfer a set of previously described microsatellite markers from other bivalve molluscs were not successful (three loci of *Lasmigona subviridis* developed by King & Robbins, GenBank accession numbers AF108124, AF108127, AF108128 and three loci of *Lampsilis abrupta* developed by Eackles & King, 2002, LabD10, LabD111, LabD213).

3.3 Material and Methods

Total DNA was extracted from foot and adductor muscle tissue from a single mussel using NucleoSpin-Tissue-Kit (Macherey-Nagel). Isolation of microsatellites was performed following Estoup *et al*. (1993) with modifications. We established two genomic libraries in order to increase the number of recombinant clones containing different inserts. For construction of a first library, total genomic DNA was digested with *Mbo*I and the resulting fragments were separated on a 1.2 % agarose gel. Fragments with 400-1,000 bp were excised, purified with NucleoSpin-Extract-Kit (Macherey-Nagel) and ligated into pUC19 vector previously digested with *Bam*H1. Construction of the second library was similar, except DNA was digested with *Tas*I and fragments were ligated into *Eco*RI digested pUC19 vector. Ligation products were transformed into *E. coli Top10* competent cells (Invitrogen) and grown on LB-Agar plates containing ampicillin, X-Gal and IPTG for selection of recombinant clones (Sambrook *et al*., 1991). A total of 4,900 recombinant white colonies were obtained from the two libraries, each transferred onto two LB-Agar master plates and incubated at 37°C overnight. One master plate was used as source for the following amplification of positively detected clones, whereas the copy master plate was required for colony lifts onto porablot NCL membranes (Macherey-Nagel). Membranes were hybridised overnight at 50°C with a mixture of 5′-DIG-labelled oligonucleotide probes $(GA)_{10}$, $(CA)_{10}$, $(CAC)_5CA$, $CT(CCT)_5$, $CT(ATCT)_6$, $(TGTA)_6TG$. A total of 85 positive clones were detected using the DIG-Nucleic-Acid-Detection-Kit (Roche). Positive clones were used as a template for PCR with universal M13 primers. Thus, we selected 34 clones containing plasmids with distinguishable insert sizes for sequencing. Plasmid-DNA from LB-broth overnight cultures was purified with NucleoSpin-Plasmid-Kit (Macherey & Nagel). Sequencing reactions were carried out using the Thermo-Sequenase-Primer-Cycle-Sequencing-Kit (Amersham Pharmacia) with universal 5′-Cy5 labelled M13 primers. Reactions were analysed on ALFexpressII DNA Analyser using ALFWIN SEQUENCE ANALYSER 2.1 software. Out of 34

sequenced clones, 32 were deemed unique. Twenty-eight clones contained microsatellites with flanking regions suitable for primer design with PRIMER3 software (Rozen & Skaletsky, 1998). Twenty primer pairs amplified products of predicted size at low rates of stuttering, and were therefore chosen for 5´-Cy5 labelling of forward primers to analyse microsatellite variability. Genotyping of 42 individuals from four different river drainages (Elbe, Main, Danube, Weser) was carried out.

PCR was performed in a total volume of 12.5 µl with the following components: 25 ng of genomic DNA, 200 nM of each primer, 0.2 mM each dNTP, 1.5 – 3.0 mM $MgCl_2$ (Table 3.1), 1x PCR buffer (10 mM Tris-HCl, 50 mM KCl, 0.08% Nonidet P40), and 0.25 U Taq DNA Polymerase (Q Biogene). PCR was carried out on a Mastercycler Gradient thermal cycler (Eppendorf) under the following cycling conditions: initial denaturation at 94°C for 4 min; 35 cycles of 94°C for 30 s, 52-56°C (Table 3.1) for 30 s, 72°C for 30 s, and final extension at 72°C for 3 min. PCR products were separated by electrophoresis on 5% denaturing 19:1 acrylamide:bisacrylamide gels on ALFexpressII DNA Analyser and scored with ALLELELINKS 1.02 software. Electrophoresis was carried out on standard plate gels with external and internal standards for exact scoring. Some alleles of locus MarMa1632 only differed by 1 bp in size but proved to be unambiguously applicable. GENEPOP3.3 software (Raymond & Rousset, 1995a) was used to generate allele frequencies, to calculate expected and observed heterozygosities (H_e, H_o) and to test the loci for genotypic disequilibrium and deficiency of heterozygotes (Fisher´s exact test).

3.4 Results and Discussion

Out of the 20 loci tested on 42 individuals from four river drainages, 13 loci were polymorphic with two to twelve alleles, averaging 6.8 alleles per locus. Their characteristics and amplification conditions are described in Table 3.1. Observed heterozygosity values (H_o) ranged from 0 to 0.850, those for expected heterozygosity (H_e) from 0.174 to 0.850. The test of genotypic disequilibrium for each pair of loci over all individuals revealed five significant values ($p < 0.00064$) for 78 comparisons after Bonferroni correction. However, linkage equilibrium was observed in all loci when testing populations separately ($p < 0.00064$). Over all populations, a significant deficiency of heterozygous genotypes was found in eight of thirteen loci, similar to the results previously described for other bivalve populations (e.g. Zouros & Foltz, 1984, Eackles & King, 2002). Tests on Hardy-Weinberg equilibrium for each

locus in each population revealed four deviations in population Elbe (MarMa3050, MarMa5167, MarMa4315, MarMa4859) and two in population Weser (MarMa3621, MarMa4859).

Since the number of available microsatellite markers for bivalve molluscs is very limited and these are the first described among the order of Unionoida, they may also be useful for screening genetic diversity in sister taxa.

Locus	GenBank Accession no.	Repeat motif	Primer sequences (5´→3´)	T_a (°C)	$MgCl_2$ (mM)	N_A	Allele size range (bp)	H_o	H_e
MarMa1632	AY255126	$(GT)_8(G)_{11}$	F: TCTGCTATGGACATGATTGT R: TCAATCAAATCAAGTTTCACC	55	3.0	8	162-170	0.350*	0.736
MarMa2671	AY255112	$(GA)_8AA(GA)_6$	F: AATTATTTCTGGACCATATGAGA R: TTACCGTATTATAGTTTTCTCTCTT	52	3.0	2	149-151	0.053	0.052
MarMa3050	AY255113	$(CA)_{14}$	F: AATACAAAGGTATCCGCATTTT R: CACAGCCTAGAATACGTATCAGAA	52	3.0	4	79-93	0.381*	0.664
MarMa3116	AY255114	$(GT)_3…(GT)_3…(CA)_5CG(CA)_{15}$	F: TTAGCCGCTATTACAAAACG R: AGAATGAAACATTCGACAGC	56	1.5	6	206-250	0.238	0.264
MarMa3621	AY255115	$(CAA)_2(GA)_{22}$	F: TGGTGTTATCCTCAACATGC R: TGGCATTCTATAAAAGTTTACCAA	55	3.0	10	173-201	0.429*	0.717
MarMa4143	AY255117	$(TC)_{16}$	F: TCAAAACCACTTGTTGTATGGT R: TGGAGCTTGTAAATAAATCCTGT	55	3.0	7	190-204	0.415*	0.712
MarMa4277	AY255118	$(CT)_{20}(CA)_{16}$	F: TGTCGGAAATCTTAGCTTGG R: GGCGAAATTGAATGCTCTAA	55	2.0	11	176-196	0.850	0.831
MarMa4315	AY255119	$(CTAT)_3(CT)_{12}(AT)_{15}AAA(CA)_7CT$ $(AT)_3$	F: TCATTGTGTCATTAGCAATTTTTG R: CCATTGCACTTAGCTGGAAA	55	2.0	10	174-220	0.512*	0.838
MarMa4322	AY255121	$(TAT)_{10}AAT(TAT)_2$	F: AAAATCACATTCATTTTCTTTCCT R: TGCCCTCAATTAAACAAAACA	55	3.0	4	200-215	0.450	0.623
MarMa4726	AY255122	$(TC)_{15}…(CA)_4$	F: CTTGTGTCCAATCCCTTTGC R: GTCAACCCATTCCAAACAAT	55	3.0	4	156-164	0.432	0.469
MarMa4859	AY255120	$(CCT)_5(CT)_{18}$	F: TGACTCCACTTTGTTCCGTTT R: TTTCGTCATTATAAATCTGGCTCA	55	3.0	12	150-184	0.406*	0.850
MarMa5167	AY255123	$(CAA)_3CAG(CAA)_1(CA)_{14}$	F: AAAGGTCGTTCTTCACATCAA R: GCAACCAAGACCCGAATTA	55	3.0	8	126-142	0.405*	0.773
MarMa5280	AY255125	$(CT)_9(CA)_9(GT)_2(AC)_3$	F: GGAACCACCTGTTTGGAAGA R: GGCCACAATTTTGAGTCCAT	55	2.0	2	198-200	0.000*	0.174

Tab. 3.1: Characteristics of 13 microsatellite loci for the freshwater pearl mussel (*Margaritifera margaritifera*): Locus designation, GenBank accession number, repeat motif (referring to sequenced allele), primer sequences (forward primers 5´Cy5-labelled), optimal annealing temperature (T_a) and $MgCl_2$ concentration, number of observed alleles (N_A), allele size range, level of observed (H_o) and expected (H_e) heterozygositiy per locus ;*indicates locus deviated from expected number of heterozygous genotypes

4 Genetic diversity and differentiation of central European freshwater pearl mussel (*Margaritifera margaritifera* L.) populations: implications for conservation and management

published: Juergen Geist, Ralph Kuehn (2005): Genetic diversity and differentiation of central European freshwater pearl mussel (*Margaritifera margaritifera* L.) populations: implications for conservation and management; *Molecular Ecology* **14**, 425-439

4.1 Abstract

Despite the fact that mollusc species play an important role in many aquatic ecosystems, little is known about their biodiversity and conservation genetics. Freshwater pearl mussel (*Margaritifera margaritifera* L.) populations are seriously declining all over Europe and a variety of conservation programmes are being established to support the remaining endangered central European populations. In order to provide guidelines for conservation strategies and management programmes, we investigated the genetic structure of 24 freshwater pearl mussel populations originating from five major central European drainages including the Elbe, Danube, Rhine, Maas and Weser, representing the last and most important populations in this area. We present a non-destructive sampling method by the collection of haemolymph for DNA analyses, which is applicable for endangered bivalves. The analyses of nine microsatellite loci with different levels of polymorphism revealed a high degree of fragmented population structure and very different levels of genetic diversity within populations. These patterns can be explained by historical and demographic effects and have been enforced by anthropogenic activities. Even within drainages, distinct conservation units were detected, as revealed from high F_{ST}-values, private alleles and genetic distance measures. Populations sampled close to contact zones between main drainage systems showed lowest levels of correct assignment to present-day drainage systems. Populations with high priority for conservation should not only be selected by means of census population size and geographic distance to other populations. Instead, detailed genetic analyses are mandatory for revealing differentiation and diversity parameters, which should be combined with ecological criteria for sustainable conservation and recovery programmes.

4.2 Introduction

Unionid bivalves are a diverse group of molluscs with a worldwide distribution (Roe & Hoeh, 2003). They play an important role in lotic and lenitic ecosystems and their presence or absence in a lake or stream has manifold implications for aquatic ecosystems (Bauer & Wächtler, 2001). Nowadays, many species suffer from severe population declines, and bivalve biodiversity is diminishing at a nearly unprecedented pace (e.g. Ricciardi & Rasmussen, 1999). One example is the freshwater pearl mussel (*Margaritifera margaritifera* L.), an indicator species for undisturbed headwater regions and small streams, which occurred in extreme densities until the middle of the 19th century, often covering the river bottoms in one or more layers. *M. margaritifera* has declined substantially throughout its holarctic range and is now highly vulnerable or threatened with extinction almost everywhere, with few populations still having a significant number of juveniles present (Cosgrove *et al.*, 2000; Young *et al.*, 2001a). Some authors even consider it to be among the most critically endangered freshwater mussels in the world (Marchordom *et al.*, 2003). Deterministic factors like pearl fishing, water pollution and eutrophication, acidification, habitat destruction, river engineering and the decline of host fish populations, have all more or less contributed to the decline. Small isolated populations, in turn, are more susceptible to the effects of inbreeding and genetic drift, which can result in reduced adaptability, survival and reproduction. Nowadays, only a few populations still exist in central Europe, mainly in the Elbe and Danube drainages, and some smaller relict populations in the Rhine/Main, Maas and Weser drainages. Pearl mussels can reach an age of more than 100 years (Bauer, 1992) and most of these populations have not been reproducing for the past 30 – 50 years.

The species is restricted to habitats with flowing waters which are low in lime and nutrients, and requires special conditions to complete its complex life cycle. Freshwater pearl mussels have separate sexes, with females being able to switch to hermaphrodites at low population densities (Bauer, 1987a). Like all freshwater mussels (Unionoidea), pearl mussels have a reproductive strategy that involves a larval “glochidia” stage, which is retained in the female brood pouch or gills and released for their intermediate stage as a parasite on a host fish before transforming into bottom-dwelling juveniles. Suitable host fishes for freshwater pearl mussels are restricted to salmonids, with a preference for brown trout (*Salmo trutta* f. *fario*) in central European populations (Wächtler *et al.*, 2001).

The vulnerability of the species requires conservation, recovery and management strategies, which include investigation of current levels of genetic diversity and differentiation within and between populations as a basis for sustainable management recommendations. Genetic studies on bivalves based on conchological convergences and parallelisms in shell shape and external morphology can be highly influenced by environmental variables such as substrate composition or water velocity (e.g. Johnson, 1970; Watters, 1994). Available allozymes and mitochondrial genes were found not to resolve genetic structures beyond species level for freshwater pearl mussels (Nagel & Badino, 2001; Marchordom *et al*., 2003). Therefore, we developed species-specific microsatellite markers for freshwater pearl mussels (Geist *et al*., 2003). Nine microsatellite markers were used in this study to reveal population diversity and differentiation among 24 central European freshwater pearl mussel populations of the five major drainages of Elbe, Danube, Rhine, Maas and Weser as a basis for ongoing species conservation efforts in these areas. The intended recovery strategies, based on semi-artificial infections of host fish, supportive breeding and the use of cultured unionids as a conservation tool underscores the need to recognize the genetic composition of natural and managed populations. To our knowledge, this is the first study on population and conservation genetics of a European freshwater bivalve, applying microsatellite markers.

4.3 Materials and Methods

Sampling strategy

A total of 558 individuals from 24 pearl mussel populations originating from five central European main drainage systems of Elbe (8 populations), Danube (8 populations), Rhine (4 populations), Maas (2 populations) and Weser (2 populations) were included in this study, representing the most important remaining pearl mussel populations of Austria, Belgium, the Czech Republic, Germany and Luxembourg (Figure 4.1). Two populations, a geographically isolated relict population (Vogelsberg, VB) and a population for which an artificial culturing technique is currently being established (Weiße Elster, WE), were also included in this study despite the fact that they consist of a few individuals only, rendering small sample numbers (4 and 6, respectively) for analyses. A description of the sampled populations, including estimates for their current census population sizes, is provided in Table 4.1. For species protection reasons, it is not allowed to provide detailed GPS-coordinates, yet they can be made available on demand by the Correspondence. Most pearl mussel populations are in

danger of extinction, which necessitates the use of a sampling method that has no negative impacts on the extant populations. Two principal sources were used for DNA-extraction in this study: Sampling of dead individuals found during river surveys (10 % of samples) and sampling of haemolymph from living specimens (90% of samples). For the latter method, mussels were removed from the river bottom and approximately 0.1 – 0.3 ml of haemolymph was collected with 1 ml syringes attached to 0,80 x 50 mm 21G x 2´´ sterican needles by gently inserting the needle into the foot of the mussels. Shells of sampled specimens were cleaned with paper towels and marked with white waterproof paint for later inspection. All mussels were then returned to their original locations within the river bed substrate. Inspection of 250 sampled mussels from 10 populations after 4 weeks, 6 months and one year revealed no mortality caused by the sampling method. Special attention was given to ensuring representative sample collection, including samples from a long river stretch in the range of mussel distribution within each river and including samples of mussels from all age classes except those with a size smaller than 4.5 cm (approximately corresponding to an age of max. 20 years). However, such young mussels only occurred in two of the investigated rivers in central Europe, from which dead individuals from younger age classes were available and included into the analyses. The sample collection was carried out from 2001 to 2003.

Drainage	Subdrainage	Population	Code	Country	N_c	Sample size
Elbe	Sächsische Saale	Zinnbach	ZI	D	7,000	26
	Sächsische Saale	Südliche Regnitz	SR	D	13,000	25
	Sächsische Saale	Wolfsbach	WB	D	2,100	24
	Sächsische Saale	Höllbach	HB	D	34,000	25
	Sächsische Saale	Mähringsbach	MB	D	11,000	25
	Sächsische Saale	Weiße Elster (Triebelbach and Rauner Bach)	WE	D	<50	6*
	Eger→Sächsische Saale	Steinselb	ST	D	16	16
	Moldau	Blanice	BL	CZ	50,000	33
Danube	Naab	Waldnaab	WN	D	3,000	26
	Naab	Biberbach	BI	D	500	25
	Regen	Wolfertsrieder Bach	WR	D	2,000	21
	Gaißa	Kleine Ohe	KO	D	7,000	32
		Ranna	RA	D	600	29
	Aschach	Leitenbach	LE	A	500	24
	Aist	Waldaist	WA	A	18,000	24
		Kamp	KA	A	23,000	24
Rhine	Weißer Main→Main	Metzlersreuther Bach	ME	D	50	26
	Fränkische Saale→Main	Schondra	SC	D	100	20
	Sauer→Mosel	Our	OU	L, D, B	1,350	27
	Mosel	Sauer	SU	B	250	26
Maas	Semois	Anlier	AN	B	1,400	26
	Semois	Rulles	RU	B	300	25
Weser	Aller	Lutter	LU	D	4,200	19
	Fulda	Vogelsberg (Ellersbach, Altefeld)	VB	D	4	4*

Tab. 4.1: Samples used for genetic analyses; N_c = estimates for census population sizes counted 1998-2003; * indicates small sample size due to small census population or sampling restrictions but expected to be representative for remaining population; A = Austria, B = Belgium, CZ = Czech Republic, D = Germany, L = Luxembourg

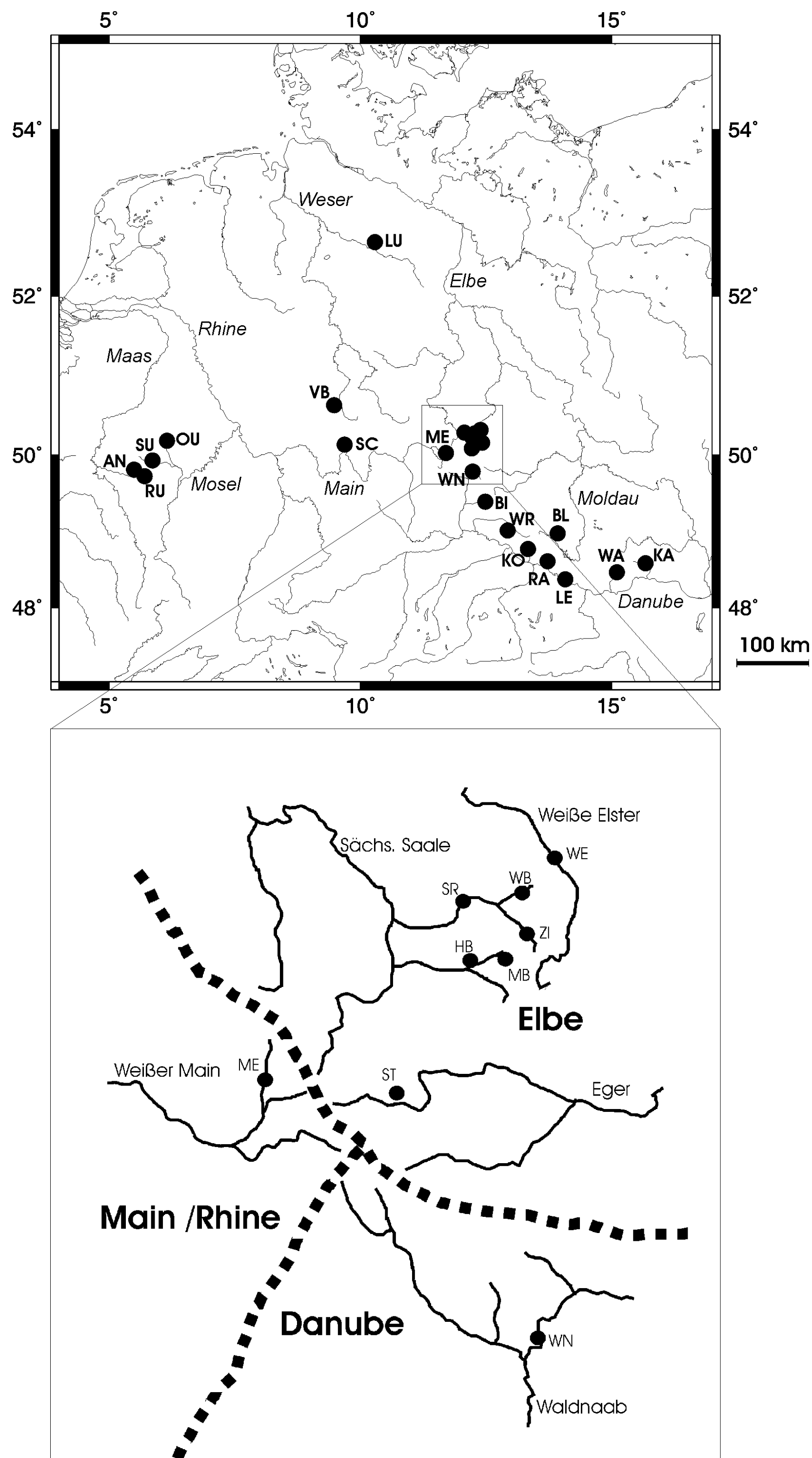


Fig. 4.1: Sampling locations (black circles) of freshwater pearl mussel (*Margaritifera margaritifera*) populations in central Europe and magnification of the sampling sites at the contact zone between the three main drainage systems of Elbe, Main/Rhine and Danube; sample codes according to table 4.1

DNA isolation and microsatellite analyses

From dead specimens, total DNA was extracted from foot and adductor mussel tissue using NucleoSpin Tissue-Kit (Macherey-Nagel), following the manufacturer´s instructions for preparation of tissue material. Haemolymph samples were transferred to 1.7 ml Eppendorf vials, cooled at 5°C and processed immediately in the laboratory. After centrifugation at 14,000g for 5 min the supernatant was discarded and DNA was isolated from the remaining cellular pellet with the NucleoSpin Tissue Kit (Machery-Nagel), as described for the tissue samples.

A total of nine microsatellite loci with different levels of polymorphism were selected for this study: Eight loci (MarMa2671, MarMa3050, MarMa3621, MarMa4143, MarMa4322, MarMa4726, MarMa5167 and MarMa5280) previously described in Geist *et al*. (2003), and one additionally developed locus MarMa5023 (GenBank accession no. AY633928). Polymerase Chain Reactions (PCRs) were performed in a total volume of 12.5 µl with the following components: 25 ng of genomic DNA, 200 nM of each primer, 0.2 mM of each dNTP, 3 mM $MgCl_2$ (2 mM $MgCl_2$ for Locus MarMa5280), 1 x PCR buffer (10 mM Tris-HCl, 50 mM KCl, 0.08% Nonidet P40), and 0.25 U *Taq* DNA Polymerase (Qbiogene). The forward primers were end-labelled with the fluorescent dye Cy5. PCR was carried out on a Mastercycler Gradient thermal cycler (Eppendorf) under the conditions described by Geist *et al*. (2003). Annealing temperature was 55°C for locus MarMa5023. PCR products were separated on 5% denaturing 19:1 acrylamide:bisacrylamide gels on ALFexpressII DNA analyser and scored with ALLELELINKS 1.02 software (Amersham Parmacia Biotech). Electrophoresis was carried out with two internal standards in each lane. Additionally, an external standard and a previously sequenced reference sample were included on each gel in order to ensure exact scoring and to facilitate cross-referencing among gels.

Statistical and population genetic analyses

GENEPOP v. 3.3 (Raymond & Rousset, 1995a) was used to calculate allele frequencies, average allele numbers per locus (A), expected and observed heterozygosities (H_e, H_o), to test the genotypic distribution for conformance with Hardy-Weinberg (HW) expectations, to test the loci for genotypic disequilibrium, to calculate pairwise F_{ST} values and to test the significance of allelic differentiation. Allelic richness (A_R) as a standardized measure of the number of alleles per locus corrected by the sample size was calculated with the FSTAT v.

2.9.3 programme package (Goudet, 2001). FSTAT v. 2.9.3 was also used to test for differences between drainages (1,000 permutations, two-sided test). Alleles were deemed as private alleles if they showed a frequency of more than 5% in one population and did not occur in any other population. Genetic distances between populations were estimated using *Nei* D_A genetic distance (Nei *et al*., 1983) as implemented in the DISPAN programme (Ota, 1993). The resulting distance matrix was used to construct a Neighbour-Joining (NJ) – phenogram in MEGA version 2 (Kumar *et al*., 1993). Bootstrap analysis was performed by first generating 1,000 distance matrices which were then used to generate 1,000 neighbour-joining trees in DISPAN (Ota, 1993). ARLEQUIN 2.0 software (Schneider *et al*., 2000) was used to hierarchically quantify genetic population structure by analysis of molecular variance (AMOVA; Excoffier *et al*., 1992), and to incorporate molecular information based on allelic frequencies. All probability tests were performed applying the Markov Chain algorithm (Guo & Thomson, 1992; Raymond & Rousset, 1995b). Sequential Bonferroni adjustments (Rice, 1989) were used to correct for multiple tests. The Bayesian approach of population assignment test (Cornuet *et al*., 1999; `as it is´ option) implemented in the GENECLASS 1.0.02 programme (Piry & Cornuet, 1999) was used to estimate the likelihood of an individual´s multilocus genotype to be assigned to the population from which it was sampled.

Relatedness between individuals was estimated based on the *F*-value from the 2MOD-programme (Ciofi & Bruford, 1999) which refers to the probability that two genes share a common ancestor within a population and correlates with effective population sizes. The 2MOD programme was also used to investigate the population history of the central European freshwater pearl mussel populations based on the coalescent theory. The method uses the comparison of the relative likelihoods of a model of immigration-drift equilibrium (gene flow model) versus drift since a certain time. A Markov Chain Monte Carlo simulation (100,000 iterations) was computed, and the first 10% of the output was discarded in order to avoid bias due to the starting conditions.

Additionally, populations were tested for recent reduction of their effective population size based on the approach of Cornuet & Luikart (1996) with the programme BOTTLENECK (Piry *et al*., 1999). The Wilcoxon sign-rank test was used to test the significance of heterozygote excess under three different models, the infinite allele model (IAM), the stepwise mutation model (SMM) and the two-phase model (TPM) with 5% multi-step changes and variance of 12, following the recommendations of Piry *et al*. (1999).

The heterozygosity contribution (*CT*) of each population to total diversity was calculated with the CONTRIB programme (Petit *et al*., 1998) by separately calculating diversity and differentiation indices measured by the expected heterozygosity. This approach allows a simultaneous comparison of populations with the average values over all populations by visualizing positive or negative *CT* % - values and supplements the genetic characterization of populations and the selection of priority populations for conservation.

4.4 Results

Linkage and Hardy-Weinberg equilibrium

The test for genotypic disequilibrium for each pair of the nine microsatellite loci over all populations gave two significant values ($p < 0.05$) for 36 comparisons (two significant values are expected by chance at the 5 % level). After Bonferroni correction for multiple tests, none of the combinations remained significant at the experimental level ($p < 0.00138$). When each population was tested separately, a linkage equilibrium between all pairs of loci was generally observed, with only few exceptions: Four significant values were found for the Waldaist population (WA) and one for the Mähringsbach (MB) population. Different loci were involved in these cases. Generally, this test implies that the genotypes of the loci used in this study segregated independently.

After Bonferroni correction, the probability test by the Markov Chain method based on the “exact Hardy-Weinberg (HW) test” of Haldane (1954) for each locus in each population showed only 5 significant deviations: Populations ST and SC at locus MarMa3621, populations WR and BL at locus MarMa4726, and population AN at locus MarMa3050.

Six populations out of 24 displayed significant deviations from the expected Hardy-Weinberg proportions after applying sequential Bonferroni correction (see Table 4.2). These deviations are not systematic; they occur at different loci (MarMa 3621, MarMa4726, MarMa5167, MarMa3050 and MarMa5023) for different populations and with a maximum of 2 deviations in the Blanice (BL) population.

Genetic diversity and relatedness within populations

An average of 7.8 alleles (standard deviation SD = 5.3) was observed for the nine microsatellite loci applied in this study. The number of alleles per locus ranged from two at loci MarMa2671 and MarMa5280 to a maximum of 16 alleles at locus MarMa5167. Allelic variation, expressed by the average number of alleles per locus (A) and allelic richness (A_R), varied strongly between and within drainage systems and was highest in the Blanice river (BL) and Wolfertsrieder Bach (WR) from the Elbe and the Danube drainage systems, respectively. A summary of the microsatellite diversity indices is provided in Table 4.2. The majority of brooks and rivers from the Elbe and Danube drainage systems tend to have a higher diversity than those from the other central European pearl mussel populations, with a few exceptions. The lowest observed values for allelic diversity (A = 1.1; A_R = 1.0) were found in the Kamp (KA) from the Danube drainage system and in the generally smaller remnant populations from the Rhine and Maas drainages, where the highest values for allelic richness are 1.6 (Metzlersreuther Bach, ME) and 1.2 (Anlier, AN). Maximum values for the average number of alleles per locus and for allelic richness were found in the Blanice (BL) population (A = 4.9; A_R = 2.1). The expected heterozygosity (H_e) per population was between 0.005 for the Kamp (KA) and 0.485 for the Blanice (BL), and the observed heterozygosity (H_o) ranged between 0.005 for the Kamp (KA) and 0.494 for the Ranna (RA), with the average H_e being 0.323 and the average H_o being 0.289 (Table 4.2).

Private alleles occurred at five different loci in six different populations and usually showed high frequencies ranging from 11.11% in the Our (OU) up to 98.08% in the Anlier river (AN). They occurred in isolated relict populations from Lutter (LU), Vogelsberg (VB) and Schondra (SC), but were also observed in drainage systems, in which other pearl mussel populations are still present. The maximum of private alleles (3) was found in the Anlier river (AN) from the Maas drainage, although it is not far from the Rulles (RU) population. One private allele was also found in the Our (OU) population, situated in the same Rhine subdrainage as the Sauer (SU) population. With exception of the highly diverse Blanice population (BL), no private alleles were detected in populations which were once connected and where a larger number of populations still exist within a small geographical range (Elbe and Danube systems).

The proportion of common ancestors within each population as inferred from the F-values of the 2MOD programme covered an extreme range from F = 0.060 in Wolfertsrieder Bach

(WR) to *F* = 0.944 and 0.942 in Kamp (KA) and Rulles (RU), respectively. The correlation between *F*-value and census population size is slightly negative ($r^2 = 0.05$ and $p = 0.288$). A low probability of common ancestors as revealed by the *F*-values was not restricted to large and dense populations such as Blanice (BL; census population size = 50,000, *F* = 0.064), in which lower rates of hermaphrodism and self-fertilization would be expected, but occasionally also occurred in populations like Steinselb (ST; *F* = 0.066), in which the total population only consisted of 16 individuals distributed over a brook section of approximately 300 m. The highest *F*-values were found in comparatively small populations of the Rhine and Maas drainages (e.g. Schondra, SC, census population size = 100, *F* = 0.856; Rulles, RU, census population size = 300, *F* = 0.942) as well as in comparatively large populations (e.g. Kamp, KA, census population size = 23,000, *F* = 0.944). On average, *F*-values were lowest in populations of the Elbe drainage followed by Danube and Weser drainages.

The Wilcoxon sign-rank test ($p < 0.05$) revealed evidence for recent bottlenecks in nine and seven populations, according to infinite allele model (IAM) and two-phase model (TPM), respectively (Table 4.2). Assuming a stepwise mutation model (SMM), however, none of the populations revealed heterozygote excess. Five populations had less than 4 polymorphic microsatellite loci and could therefore not be tested.

The heterozygosity contribution (*CT*) of each population to total diversity is shown in Figure 4.2, which demonstrates the large differences in diversity and differentiation of populations. Highest diversity contributions were observed in regions with a large number of remaining populations (Elbe and Danube systems). From the smaller populations of the Rhine, Maas and Weser catchments, only the Lutter (LU) population, which is still reproducing, showed a positive heterozygosity contribution with respect to diversity. The two most downstream Danubian populations of Waldaist (WA) and Kamp (KA), and the small Schondra (SC), Sauer (SU), Anlier (AN) and Rulles (RU) populations showed the most negative values for diversity contribution.

Population	N	A	A_R	A_P	f_P	H_e	H_o	P_{HW}	F	$HE_{(IAM/TPM/SMM)}$
Elbe										
ZI	26	2.9	1.8	-		0.381	0.372	n.s.	0.299	+ / + / -
SR	25	3.0	1.8	-		0.393	0.400	n.s.	0.295	- / - / -
WB	24	1.9	1.5	-		0.254	0.245	n.s.	0.448	- / - / -
HB	25	3.6	2.0	-		0.448	0.418	n.s.	0.156	+ / + / -
MB	25	3.7	1.9	-		0.441	0.413	n.s.	0.095	+ / - / -
WE	6	2.6	1.9	-		0.436	0.278	n.s.	0.133	- / - / -
ST	16	3.4	2.0	-		0.447	0.361	*	0.066	- / - / -
BL	33	4.9	2.1	1	14.06	0.485	0.418	**	0.064	- / - / -
average	*22.5*	*3.236*	*1.9*	*0.125*		*0.411*	*0.363*		*0.195*	
Danube										
WN	26	3.1	1.9	-		0.415	0.385	n.s.	0.164	+ / + / -
BI	25	3.0	2.0	-		0.461	0.489	n.s.	0.278	+ / + / -
WR	21	4.0	2.1	-		0.531	0.460	***	0.060	+ / - / -
KO	32	2.9	1.9	-		0.424	0.369	n.s.	0.248	+ / + / -
RA	29	3.3	2.0	-		0.479	0.494	n.s.	0.216	+ / + / -
LE	24	3.7	2.0	-		0.480	0.449	n.s.	0.218	- / - / -
WA	24	2.7	1.4	-		0.176	0.163	n.s.	0.389	- / - / -
KA	24	1.1	1.0	-		0.005	0.005	n.d.	0.944	n.d.
average	*25.6*	*2.972*	*1.8*	*0.000*		*0.371*	*0.352*		*0.315*	
Rhine										
ME	26	2.1	1.6	-		0.313	0.325	n.s.	0.560	+ / + / -
SC	20	1.6	1.2	1	97.50	0.081	0.023	***	0.856	n.d.
OU	27	1.8	1.3	1	11.11	0.184	0.123	n.s.	0.685	- / - / -
SU	26	1.3	1.2	-		0.082	0.038	*	0.860	n.d.
average	*24.8*	*1.695*	*1.3*	*0.500*		*0.165*	*0.127*		*0.740*	
Maas										
AN	26	1.7	1.2	3	98.08 78.85 12.00	0.107	0.062	***	0.656	n.d.
RU	25	1.1	1.1	-		0.052	0.044	n.d.	0.942	n.d.
average	*25.5*	*1.389*	*1.2*	*1.500*		*0.080*	*0.053*		*0.664*	
Weser										
LU	19	2.6	1.8	1	41.67	0.393	0.412	n.s.	0.385	- / - / -
VB	4	1.9	1.6	1	33.33	0.288	0.185	n.s.	0.451	- / - / -
average	*11.5*	*2.222*	*1.7*	*1*		*0.341*	*0.299*		*0.418*	
Total average	***23.3***	***2.7***	***1.7***			***0.323***	***0.289***		***0.395***	

Tab. 4.2: Microsatellite diversity indices for central European freshwater pearl mussel (*Margaritifera margaritifera*) populations. Sample size (N), average number of alles per locus (A), mean allelic richness per population (A_R), number of private alleles (A_P), frequency of private alleles (f_P), expected (H_e) and observed (H_o) heterozygosity, result of Hardy-Weinberg probability test for deviation from expected Hardy-Weinberg proportions (P_{HW}), F-value based on the 2MOD programme, and test of heterozygosity excess (*HE*) using Wilcoxon sign-rank test based on infinite allele model (IAM), two-phased model (TPM) and stepwise mutation model (SMM)

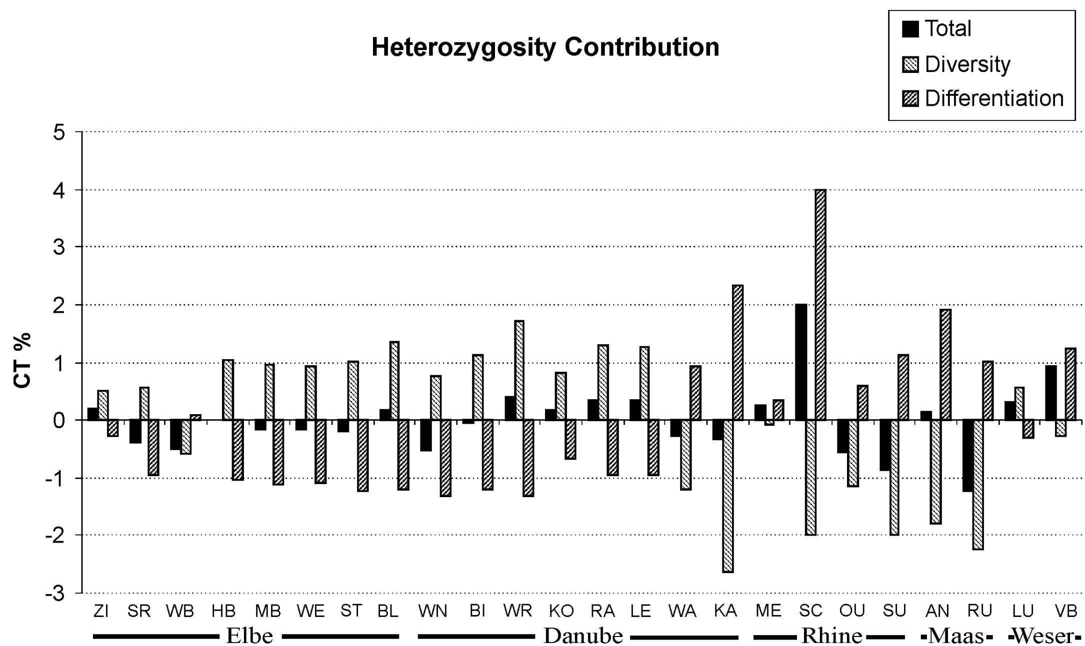


Fig. 4.2: Heterozygosity contribution *CT* to total diversity (subdivided into a diversity and a differentiation compound) for 24 central European freshwater pearl mussel (*Margaritifera margaritifera*) populations based on CONTRIB-calculations according to Petit *et al.* (1998)

Genetic differentiation between populations

The microsatellite markers applied in this study reveal a high degree of genetic differentiation among most of the remaining central European freshwater pearl mussel populations with an overall average F_{ST}-value of 0.374 (SD = 0.23). Pairwise F_{ST}-values ranged from 0.001 between the geographically adjacent populations of Steinselb (ST) and Höllbach (HB) to values as high as 0.940 between the geographically very distant populations of Rulles (RU) from the Rhine drainage and Kamp (KA) from the most downstream Danubian pearl mussel tributary. The differences in genotype frequencies were highly significant ($p < 0.001$) for most pairwise comparisons of populations (Table 4.3).

F_{ST}-values differ significantly ($p = 0.036$) within drainages and are on average highest for Maas ($F_{ST} = 0.773$), followed by the Rhine ($F_{ST} = 0.645$) and the Weser ($F_{ST} = 0.369$). For populations belonging to the Elbe and Danube system, F_{ST}-values are comparatively low, with $F_{ST} = 0.121$ and 0.240, respectively.

AMOVA analyses of hierarchical gene diversity revealed that 58% of the genetic variation was accounted within individuals, 5% among individuals within populations and 37% among populations. The global fixation indices were 0.079, 0.374 and 0.423 for F_{IS}, F_{ST} and F_{IT}, respectively.

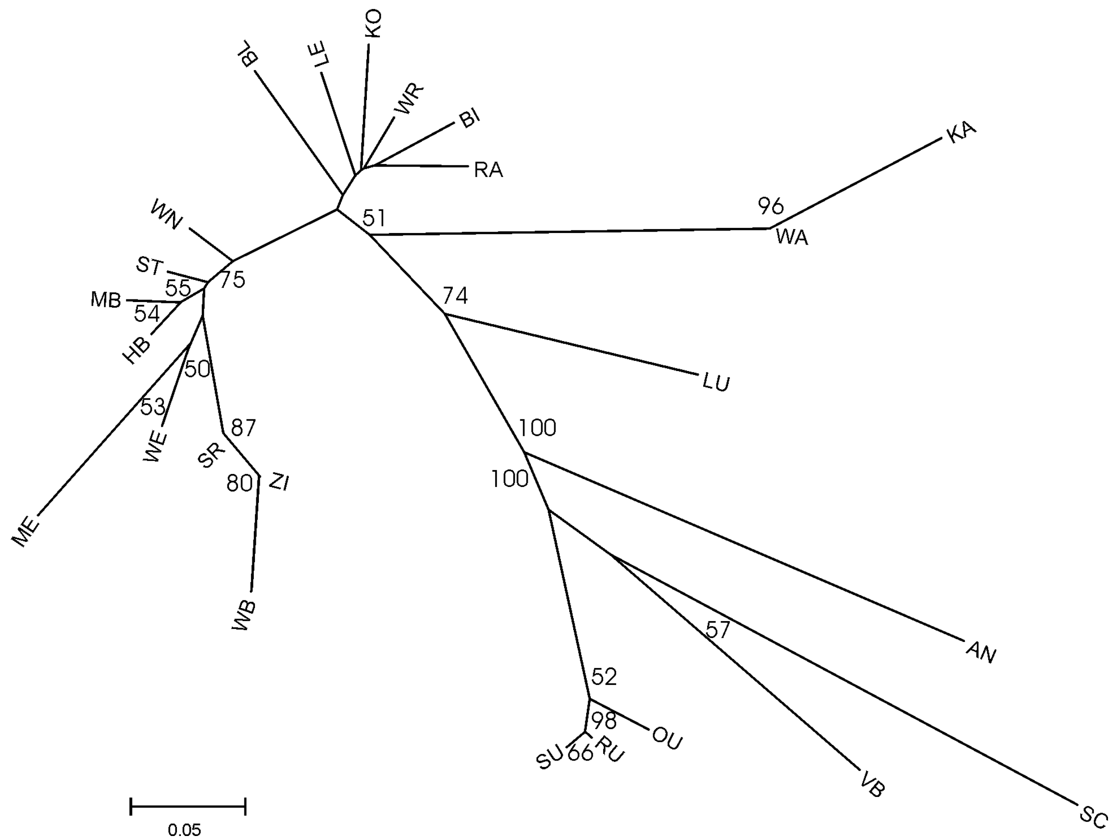


Fig. 4.3: Neighbour-Joining (NJ) phenogram based on *NeiD$_A$* (Nei *et al.* 1983) genetic distance for central European freshwater pearl mussel populations. Numbers indicate nodes with bootstrap support of more than 50% for 1,000 replications

The Neighbour-Joining (NJ) phenogram depicting the underlying structure of the *Nei D_A*-distance matrix illustrates the high degree of genetic differentiation between the populations, and reveals that the observed genetic structure does not necessarily match with drainages at present (Figure 4.3). For instance, the Anlier (AN) and Rulles (RU) populations are quite clearly separated with long branchlengths in the NJ-dendrogram, supported by high bootstrap values, despite the fact that both belong to the Maas drainage and that their geographical distance is only 20 km of river length. Danubian populations do not cluster together either, but split in a South-Eastern group (Waldaist, WA and Kamp, KA), a central Danubian group (Leitenbach, LE; Kleine Ohe, KO; Wolfertsrieder Bach, WR; Biberbach, BI and Ranna, RA), and a northernmost Danubian population (Waldnaab, WN). In the contact zone of the three main drainage systems of Main/Rhine, Elbe and Danube in Northern Bavaria, the separation

of populations from different drainages is not evident from the NJ-dendrogram. For instance, populations from today's northernmost Danube drainage (Waldnaab, WN) and from the upstream Main/Rhine drainage (Metzlersreuther Bach, ME) both cluster closer to the geographical adjacent Elbe populations. Similarly, the Blanice (BL) population from the eastern part of the Bavarian forest clusters together with the geographically adjacent Danubian populations instead of grouping together with other Elbe populations. In the contact zone of Maas and Rhine drainages, the Rulles (RU) population from the Maas drainage clusters to the adjacent Rhine populations Sauer (SU) and Our (OU). These results are supported by assignment tests (Table 4.4). An average of 79.4% (ranging from 38% to 100%) of the individuals was correctly assigned to its population of origin and a higher percentage of 93.0% (ranging from 65% to 100%) was correctly assigned to its drainage of origin at present. The lowest levels of correct assignment to the present-day drainage system mostly occurred in populations which are situated in the contact zones with adjacent drainages (e.g. Steinselb, ST; Blanice, BL; Waldnaab, WN; Sauer, SU). The lowest levels of correct assignment to specific rivers within certain drainages were found for populations that once were or still are connected. For instance, in the interconnected Zinnbach-Wolfsbach-Südliche Regnitz system (see Figure 4.1), out of 26 individuals analysed from Zinnbach (ZI), 58% are correctly assigned to their brook of origin, 23% are assigned to the Wolfsbach (WB) and 15% to the Südliche Regnitz (SR). In one case (Waldaist, WA), more than 50% of the individuals were assigned to an adjacent population.

Populations with 100% levels of correct assignment to their rivers of origin (Kamp, KA; Metzlersreuther Bach, ME; Schondra, SC; Anlier, AN; Lutter, LU; Vogelsberg, VB) can be considered to be genetically distinct and show long branches in the NJ-dendrogram with highly supported bootstrap-values. In most cases, their uniqueness is supported by private alleles as well. However, there are also populations with private alleles (Blanice, BL and Sauer, SU), which neither yield high values in the assignment tests nor appear as clearly separate and well-supported branches in the dendrogram.

The heterozygosity contribution to differentiation (Figure 4.2) reflects the above described results and shows that genetically variable populations from the Elbe and Danube drainage are usually those with low differentiation indices, whereas populations with a low genetic variability from Rhine, Maas and Weser catchment are those with the highest differentiation

indices. The two populations from Waldaist (WA) and Kamp (KA) show a remarkable genetic contribution.

Based on the results of the 2MOD programme (Ciofi & Bruford, 1999), the strong differentiation of the pearl mussel populations suggests a low level of gene flow between the extant populations. The relative likelihood of the model of gene flow – drift equilibrium versus drift revealed a drift-model for the central European freshwater pearl mussel populations ($p = 1.0$).

Population	Elbe								Danube								Rhine				Maas		Weser	
	ZI	SR	WB	HB	MB	WE	ST	BL	WN	BI	WR	KO	RA	LE	WA	KA	ME	SC	OU	SU	AN	RU	LU	VB
ZI (Zinnbach)		0.026	0.025	0.092	0.102	0.128	0.136	0.196	0.126	0.221	0.213	0.185	0.332	0.309	0.433	0.506	0.277	0.995	0.569	0.624	0.618	0.617	0.354	0.658
SR (Südl. Regnitz)	0.039***		0.038	0.039	0.048	0.048	0.052	0.139	0.056	0.170	0.142	0.163	0.216	0.224	0.328	0.396	0.173	0.892	0.431	0.461	0.457	0.451	0.275	0.604
WB (Wolfsbach)	0.058***	0.081***		0.146	0.135	0.144	0.173	0.226	0.167	0.205	0.254	0.246	0.394	0.339	0.330	0.386	0.377	0.870	0.552	0.609	0.532	0.593	0.418	0.674
HB (Höllbach)	0.111***	0.051***	0.210***		0.014	0.037	0.003	0.148	0.018	0.149	0.103	0.138	0.135	0.182	0.364	0.446	0.129	0.915	0.434	0.475	0.516	0.470	0.263	0.627
MB (Mähringsbach)	0.122***	0.062***	0.200***	0.016***		0.032	0.017	0.137	0.028	0.151	0.088	0.142	0.170	0.200	0.329	0.411	0.169	0.873	0.388	0.403	0.456	0.395	0.208	0.578
WE (Weiße Elster)	0.157***	0.064**	0.252***	0.038**	0.031**		0.041	0.159	0.069	0.133	0.116	0.128	0.171	0.229	0.324	0.395	0.148	0.760	0.409	0.435	0.398	0.405	0.245	0.598
ST (Steinselb)	0.155***	0.067***	0.248***	0.001	0.018**	0.032		0.099	0.006	0.148	0.084	0.151	0.117	0.136	0.375	0.469	0.100	0.783	0.333	0.371	0.461	0.369	0.203	0.542
BL (Blanice)	0.188***	0.142***	0.257***	0.134	0.128***	0.134***	0.092***		0.096	0.138	0.087	0.094	0.129	0.070	0.398	0.510	0.164	0.690	0.272	0.298	0.490	0.282	0.189	0.295
WN (Waldnaab)	0.151***	0.075***	0.238***	0.023***	0.034***	0.081***	0.006**	0.100***		0.112	0.069	0.113	0.115	0.121	0.329	0.413	0.159	0.751	0.325	0.362	0.427	0.341	0.199	0.435
BI (Biberbach)	0.217***	0.175***	0.260***	0.143***	0.146***	0.132***	0.140***	0.124***	0.120***		0.069	0.124	0.103	0.101	0.174	0.244	0.413	0.542	0.313	0.357	0.367	0.305	0.295	0.344
WR (Wolferstr. B.)	0.194***	0.141***	0.278***	0.094***	0.084***	0.093***	0.073***	0.074***	0.074***	0.065***		0.072	0.032	0.040	0.212	0.284	0.238	0.774	0.334	0.350	0.503	0.328	0.207	0.464
KO (Kleine Ohe)	0.197***	0.178***	0.296***	0.142***	0.147***	0.140***	0.151***	0.097***	0.128***	0.130***	0.075***		0.146	0.120	0.369	0.448	0.241	0.794	0.524	0.556	0.597	0.499	0.262	0.514
RA (Ranna)	0.273***	0.202***	0.361***	0.128***	0.155***	0.157***	0.109***	0.115***	0.119***	0.100***	0.031***	0.144***		0.045	0.288	0.371	0.210	0.835	0.310	0.329	0.531	0.304	0.249	0.467
LE (Leitenbach)	0.262***	0.207***	0.340***	0.161***	0.174***	0.188***	0.123***	0.066***	0.124***	0.098***	0.036***	0.122***	0.046***		0.293	0.385	0.273	0.688	0.315	0.363	0.589	0.339	0.289	0.368
WA (Waldaist)	0.477***	0.417***	0.506***	0.410***	0.395***	0.486***	0.438***	0.393***	0.405***	0.274***	0.294***	0.415***	0.345***	0.357***		0.004	0.730	0.896	0.577	0.585	0.472	0.523	0.560	0.752
KA (Kamp)	0.624***	0.578***	0.688***	0.564***	0.555***	0.782***	0.629***	0.534***	0.566***	0.449***	0.465***	0.557***	0.497***	0.525***	0.079***		0.856	1.026	0.703	0.710	0.533	0.638	0.666	0.920
ME (Metzlersr. B.)	0.312***	0.227***	0.440***	0.170***	0.208***	0.218***	0.146***	0.189***	0.207***	0.353***	0.241***	0.265***	0.230***	0.274***	0.613***	0.747***		1.394	0.502	0.494	0.717	0.501	0.280	0.681
SC (Schondra)	0.651***	0.634***	0.725***	0.603***	0.601***	0.713***	0.616***	0.516***	0.591***	0.513***	0.546***	0.583***	0.561***	0.545***	0.791***	0.939***	0.732***		0.534	0.627	0.681	0.566	0.745	0.467
OU (Our)	0.527***	0.471***	0.601***	0.443***	0.426***	0.516***	0.418***	0.327***	0.402***	0.377***	0.371***	0.481***	0.360***	0.372***	0.665***	0.820***	0.546***	0.710***		0.028	0.388	0.043	0.274	0.228
SU (Sauer)	0.610***	0.554***	0.695***	0.527***	0.502***	0.661***	0.524***	0.399***	0.492***	0.469***	0.449***	0.551***	0.432***	0.465***	0.752***	0.915***	0.617***	0.835***	0.154***		0.404	0.012	0.281	0.227
AN Anlier)	0.592***	0.535***	0.654***	0.525***	0.508***	0.601***	0.543***	0.474***	0.506***	0.457***	0.494***	0.551***	0.502***	0.534***	0.699***	0.872***	0.660***	0.821***	0.651***	0.761***		0.350	0.423	0.563
RU (Rulles)	0.627***	0.569***	0.714***	0.543***	0.517***	0.701***	0.549***	0.404***	0.499***	0.458***	0.457***	0.549***	0.433***	0.471***	0.763***	0.940***	0.640***	0.858***	0.243***	0.132**	0.773***		0.264	0.179
LU (Lutter)	0.320***	0.269***	0.421***	0.237***	0.205***	0.236***	0.201***	0.175***	0.206***	0.254***	0.180***	0.246***	0.219***	0.242***	0.535***	0.697***	0.313***	0.628***	0.394***	0.482***	0.541***	0.496***		0.434
VB (Vogelsberg)	0.473***	0.452***	0.599***	0.406***	0.396***	0.396***	0.373***	0.232***	0.367***	0.294***	0.292***	0.380***	0.324***	0.287***	0.691***	0.936***	0.530***	0.734***	0.422***	0.619***	0.732***	0.669***	0.381***	

Tab. 4.3: Pairwise estimates of F_{ST} between central European freshwater pearl mussel (*Margaritifera margaritifera*) populations *p<0.05; **p<0.01;*p<0.001 (below diagonal) and *NeiD$_A$* (Nei *et al*., 1983) distances (above diagonal)**

Population	Elbe								Danube								Rhine				Maas		Weser		All
	ZI	SR	WB	HB	MB	WE	ST	BL	WN	BI	WR	KO	RA	LE	WA	KA	ME	SC	OU	SU	AN	RU	LU	VB	
ZI (Zinnbach)	**15**	2	1					1																	19
SR (Südl. Regnitz)	4	**19**	1			1																			25
WB (Wolfsbach)	6	2	**22**					1																	31
HB (Höllbach)	1	2		**14**	2		1		2		1														23
MB (Mähringsbach)				3	**18**		1		1																23
WE (Weiße Elster)				1	3	**4**	2																		10
ST (Steinselb)				3			**9**		2																14
BL (Blanice)								**25**							1										26
WN (Waldnaab)				3	2		3	1	**20**		1	2													32
BI (Biberbach)								1	1	**23**			1												26
WR (Wolferstr. B.)											**16**		2	2											20
KO (Kleine Ohe)								1			1	**28**	1												31
RA (Ranna)								1		1	1	1	**23**												27
LE (Leitenbach)								1		1		1	2	**22**											27
WA (Waldaist)											1				**9**										10
KA (Kamp)															14	**24**									38
ME (Metzlersr. B.)				1		1											**26**								28
SC (Schondra)																		**20**							20
OU (Our)																			**20**	1					21
SU (Sauer)																			4	**16**		4			24
AN (Anlier)																					**26**				26
RU (Rulles)																			3	9		**21**			33
LU (Lutter)																							**19**		19
VB (Vogelsberg)								1																**4**	5
Sample size	26	25	24	25	25	6	16	33	26	25	21	32	29	24	24	24	26	20	27	26	26	25	19	4	558
Observed number assigned to sample site	15	19	22	14	18	4	9	25	20	23	16	28	23	22	9	24	26	20	20	16	26	21	19	4	443
Percent correctly assigned to sample site	58	76	92	56	72	67	56	76	77	92	76	88	79	92	38	100	100	100	74	62	100	84	100	100	79.4
Observed number assigned to main drainage of origin	26	25	24	21	23	5	13	27	21	25	20	32	29	24	23	24	26	20	24	17	26	21	19	4	519
Percent correctly assigned to drainage of origin	100	100	100	84	92	83	81	82	80	100	95	100	100	100	96	100	100	100	89	65	100	84	100	100	93.0

Tab. 4.4: Assignment test for freshwater pearl mussel (*Margaritifera margaritifera*) based on the Bayesian Method (`as it is´ option) implemented in the GENECLASS 1.0.02 programme (Piry & Cornuet, 1999)

4.5 Discussion

Population structure

The results of the microsatellite analyses clearly reveal a high degree of population substructure among extant central European pearl mussel populations. They also show that diversity within pearl mussel populations differs strongly and only slightly correlates with census population size. Differences in genetic variation can generally be explained by (i) disequilibrium of mutation and selection connected with the evolutionary history of populations, and (ii) disequilibrium of drift and migration linked with the effects of fragmentation of populations and their demographic background. Detailed genetic analyses are required for the identification of priority populations for conservation with respect to their uniqueness in terms of genetic divergence from other populations and regarding their genetic diversity. Microsatellites, with their high resolution, are the markers of choice for these investigations of pearl mussel populations. The use of shell morphology characters can be deceptive when describing differentiation among mussel populations, as these characters largely depend upon environmental variables (e.g. Johnson, 1970; Watters, 1994). In fact, the use of ecophenotypic characters has led to a confusing number of contentious or uncertain taxa of lesser rank among pearl mussels and has produced confused or disputed taxonomies (Chesney & Oliver, 1998) which can result in poor conservation strategies. However, in some cases morphologically atypical mussels (e.g. those from Schondra, SC and Rulles, RU) showed a strong genetic divergence to other populations. For the majority of populations, a link between genetic status and shell shape was not evident, underscoring the strong influence of environmental variables on these characters.

With respect to the taxonomic insufficiency and disputed taxonomy of ecophenotypes among freshwater pearl mussel populations, we use the term conservation unit (CU) as defined by Moritz (2002), Luck *et al*. (2003) and Manel *et al*. (2003) for a population or a group of populations that it is important to conserve. The conservation goals attributed to the concept of CUs for freshwater pearl mussel populations involve maintaining genetic diversity in the species, combining concepts of minimum viable populations (Soulé, 1987; Nunney & Campbell, 1993), evolutionary significant units, ESUs (Moritz, 1994; Crandall *et al*., 2000), and management units, MUs (Moritz, 1994).

The genetic diversity and differentiation of pearl mussel populations found in this study can be explained by different factors, including colonisation from different glacial refugia, post-glacial recolonisation and the generally complex colonisation of new habitats due to the specificity between pearl mussel glochidia and their narrow spectrum of host fish vectors. Population structure is additionally influenced by the fact that the species reveals a specialization on clear and cold streams of the trout region with low levels of nutrients and lime, limiting the potential geographical distribution range. Moreover, anthropogenic factors like habitat alteration, water pollution effects and destructive pearl fishing have driven many populations to extinction or left small fragmented remnant populations. The current population structure of pearl mussel populations can thus be described as an anthropogenic fragmented metapopulation, showing stronger susceptibility to the loss of genetic variability and risk of extinction than other population structures. This explanation is also supported by the results of the model of gene flow – drift equilibrium versus drift, which revealed predominant drift effects and by the fact that significant bottleneck effects were detected in many populations.

Additionally, our study shows that present-day population differentiation does not always match with present-day drainage systems, revealing the complex pattern of pre- and postglacial colonisation in the contact zones of drainage systems. This effect can most likely be explained by historical changes in the flow direction of individual tributaries towards different drainages, postglacial effects and the temporal connections between different drainage systems at those times (for details see Hantke, 1993). In contrast to our results, allozyme data for the cold-adapted bullhead (*Cottus gobio*) showed a marked genetic differentiation across drainage basins in the contact zone of Elbe, Danube and Main/Rhine in Northern Bavaria (Hänfling & Brandl, 1998). These differences can be most likely explained by different dispersal and colonisation patterns between bullhead and brown trout (*Salmo trutta*) as the host fish vector for pearl mussels. Data on the genetic structure of the much more dispersing brown trout would be more conclusive in this respect. Genetic studies of brown trout (e.g. Bernatchez, 2001; Weiss *et al*., 2001), however, do not match with the distribution and sampling pattern of pearl mussels investigated in this study.

Distinct conservation units (CUs) for freshwater pearl mussel populations are not restricted to different drainages. Simultaneously, CUs are found within drainage systems. For instance, the Danubian drainage system is subdivided into three groups: A southern Danubian cluster of

Austrian Waldaist (WA) and Kamp (KA) populations, a cluster of central populations and the most upstream Waldnaab (WN) population, which groups with the Elbe populations.

The analyses of genetic diversity revealed significant differences both between drainages and between populations within drainage systems. Low levels of genetic diversity within certain populations can be the result of the fragmented metapopulation structure, implying founder effects and bottlenecks. According to IAM and TPM, recent bottlenecks were detected in populations from the drainages of Rhine, Elbe and Danube. The high number of monomorphic microsatellite loci in five other populations (KA, SC, SU, AN, RU) prevented them from being tested for excess of heterozygotes with the BOTTLENECK approach. The high numbers of monomorphic loci, together with the high F-values, suggest that bottlenecks may also have had predominant effects in these populations. The fact that all Danubian populations in Bavaria showed heterozygote excess could be explained by recent anthropogenic influences, as all of the populations in this area were intensively exploited after the regal right to harvest pearl mussels was abolished in this region in the year 1874 (Meißner, 1912). Furthermore, the species´ extraordinary life cycle suggests a higher likelihood for the effects of small populations such as inbreeding and drift. The ability of female pearl mussels to switch to hermaphrodites at low densities of males and the enormous reproduction potential of single individuals (Bauer, 1987a) can to some extent explain the comparatively low measures of genetic diversity accomplished by high census population sizes. However, the species´ reproduction strategy suggests that pearl mussels may be less susceptible to inbreeding depression than other species. In fact, a viable and well reproducing population from Portugal (Geist, pers. obs.) shows very low levels of genetic variability. However, the only two populations included in this study which still show high levels of reproduction (Blanice, BL and Lutter, LU) are among those with the highest intrapopulation diversity indices. Within interconnected river systems with extant pearl mussel populations in different tributaries (e.g. Zinnbach – Wolfsbach – Südliche Regnitz), genetic diversity was usually observed to be lowest in the smallest headwater streams, in which recent population bottlenecks were detected with higher probability (e.g. Zinnbach). This observation could be explained by factors of environmental stochasticity, like the higher risk for small headwater tributaries to fall dry during summer or freeze completely during winter. In this case, extinction and recolonisation led to the observed lower indices of genetic variability.

Conservation and management implications

When implying sustainable conservation management and recovery strategies for freshwater pearl mussel populations, the loss of genetic diversity should be minimized by retaining the CUs. First, it is the distinctiveness and differentiation of a population by comparison with other extant populations in terms of its allelic composition. Populations that are characterized by an independent evolutionary history, as indicated by private alleles, high F_{ST}-values, long branches with high bootstrap support in the phenogram, and a low percentage of misclassification in the assignment test, can be considered as separate conservation units (CUs), as in the case of Lutter (LU), Vogelsberg (VB), Schondra (SC), Metzlersreuther Bach (ME), Anlier (AN) and Our (OU). Within the Danubian drainage, three different CUs can be defined: A downstream group comprising Waldaist (WA) and Kamp (KA), a central Danubian group (Leitenbach, LE; Kleine Ohe, KO; Ranna, RA; Biberbach, BI; Wolfertsrieder Bach, WR) and the northernmost Waldnaab (WN) unit. Elbe populations can be subdivided into two CUs, a Northern Bavarian group (Steinselb, ST; Mähringsbach, MB; Höllbach, HB; Weiße Elster, WE; Südliche Regnitz, SR; Zinnbach, ZI; Wolfsbach, WB) and the separate Czech Blanice (BL) population. This also implies that no stocking attempts with mussels or glochidia from other distinct CUs should be carried out within these populations as long as individuals from the original populations are still present. The maintenance of several isolated populations can actually increase overall genetic diversity, because allelic differences can be preserved due to local adaption to different habitats.

Adaptive differences between CUs due to different natural selection pressures may occur, despite the fact that differentiation between populations is additionally enhanced by drift effects. Mixing with other populations could thus result in outbreeding depression, i.e. the reduction in fitness caused by the breakdown of coadapted gene complexes (Templeton, 1986). There were several unsuccessful attempts in Germany to found new populations by translocating mussels from one river to other rivers (Scherf, 1980). Other studies revealed a one-year survival rate of only 50% for inter river transfers of pearl mussels in Finland (Valovirta, 1990). Despite the fact that other reasons cannot be ruled out in these cases, both observations indicate local adaption of pearl mussels to specific habitats and suggest that it is important to recognise CUs in pearl mussel conservation. With exception of the Waldaist (WA), the results of the assignment test can be well explained by natural evolutionary (colonisation/demography) patterns. It has to be mentioned, however, that possible historical

stocking activities with mussels from populations that are nowadays extinct could not be detected in this study.

Despite the recommendation to manage distinct CUs separately, it is essential to minimize the loss of genetic diversity within populations, as loss of genetic heterozygosity can have deleterious effect on population fitness (e.g. Reed & Frankham, 2003). Conservation management and recovery strategies such as semi-artificial breeding and culturing techniques, have to balance between maintenance of genetic divergence and diversity. A drift-migration equilibrium, as it can be achieved by rotation crossing (Kimura & Crow, 1963), would ideally meet these criteria. It has to be considered, however, that freshwater pearl mussels, with the ability of females to switch to hermaphrodites at low population densities (Bauer, 1987a) are probably better adapted to inbreeding effects than other animal species.

For supportive breeding in interconnected river systems, like those of Zinnbach (ZI), Wolfsbach (WB) and Südliche Regnitz (SR) (see Figure 4.1), it would be sufficient to collect glochidia from Südliche Regnitz and subsequently release them to the upstream tributaries ZI and WB, because genetic variability is highest in the most downstream SR and no other alleles are found in the upstream tributaries of ZI and WB.

Management guidelines can be recommended, according to a classification of extant pearl mussel populations into four separate categories: Large populations with high genetic diversity, small populations with high diversity, large populations with low diversity and small populations with low diversity.

In general, large populations with high diversity indices such as Blanice (BL) seem to have been consistently stable or to have fluctuated at high population densities with high levels of intrapopulation gene flow, low levels of hermaphrodism and no recent bottlenecks. It is also likely that such populations have had high densities of host fish and large areas of suitable substrate for the development of juveniles, allowing a diversity of offspring from different parent mussels to grow up naturally and continuously. From the conservation point of view, they are probably less susceptible to be driven to extinction than other populations and habitat protection can be considered to be the most important conservation tool.

High diversity indices in small populations like Steinselb (ST) can probably be explained as being historically intact and large populations, that have faced a severe recent decrease, due to anthropogenic deterministic effects of habitat destruction, water pollution or over-exploitation, that are not linked to genetic selection. Bottleneck effects, however, would probably be detected in the offspring of these populations and can be avoided by applying breeding strategies on a genetic basis. Such populations deserve high priority in conservation and should be recovered as quickly as possible, in order to avoid the effects of genetic stochasticity on small populations. In areas in which genetically closely related populations from the same CU are still available (e.g. central Danubian CU), gene flow between these populations may be advantageous.

In contrast, the genetic status of large populations with low diversity levels and low effective population sizes like Kamp (KA) can most likely be explained by colonisation with few founder individuals or pronounced population bottlenecks in the past, followed by a subsequent recovery. Management strategies in such populations should try to maintain diversity by selecting genetically different parental individuals. Small populations with low levels of diversity (like Schondra, SC or Rulles, RU) seem to be relict populations that have been isolated for quite a long time, probably characterized by a continuous decline in genetic diversity over a long period. Special concern should be attributed to avoid further loss of genetic diversity in these populations when imploying artificial breeding and culturing techniques.

4.6 Conclusions

Our data show that detailed genetic analyses are mandatory for selecting priority populations for conservation because (i) genetic differentiation does not always correlate with geographical distance, i.e. populations with private alleles and high F_{ST}-values can occur even within drainage systems, and (ii) actual census population sizes only weakly correlate with F-values ($r^2 = 0.05$ and $p = 0.288$), i.e. present-day large populations are not necessarily those with high diversity levels and effective population sizes. Thus, from a genetic point of view, a sound and effective management strategy cannot only focus on the protection and the support of comparatively large remaining populations from geographically distinct areas.

The issue of defining conservation and management strategies for freshwater pearl mussel populations clearly illustrates the challenges involved in conservation of endangered species, and is closely connected with the problem of choosing a single large refuge rather than several small refuges in island biography, the so-called SLOSS controversy (Simberloff & Aberle, 1982). Sustainable management and recovery of pearl mussel populations can benefit from a combined approach, integrating applications of ecological science with the selection of priority populations based on genetic criteria for differentiation and diversity.

5 The potential of using mollusc shells for DNA-based molecular analyses

submitted: Juergen Geist, Ralph Kuehn: The potential of using mollusc shells for DNA-based molecular analyses

5.1 Scope of using mollusc shells for DNA-analyses

Many mollusc species are critically endangered but often little is known about their phylogeny and population genetics. Mollusc shells can provide valuable sources for DNA-based studies and shells are often available from museum collections or non-invasive field-sampling. Despite this great potential of using shell material for DNA-based analyses, several aspects must be considered to optimise the results and to avoid pitfalls.

5.2 Introduction

Every body cell contains an organism's complete genetic information. During the process of mollusc shell formation, cells of the shell forming tissue and their DNA become embedded and preserved in carbonate shell material. The methodology of analysing shell DNA samples is similar to the use of semi-destructive (antlers, bones, teeth, horn, sloughed skin), non-destructive and non-invasive (hair, feather, scat, urine) samples and the analysis of ancient DNA (aDNA). Even from partly degraded mollusc shells, DNA-based studies are possible (Figure 5.1). Shell material can thus enlarge the suite of available sampling material from endangered molluscs, others being tissue from dead specimen, and haemolymph from living individuals.

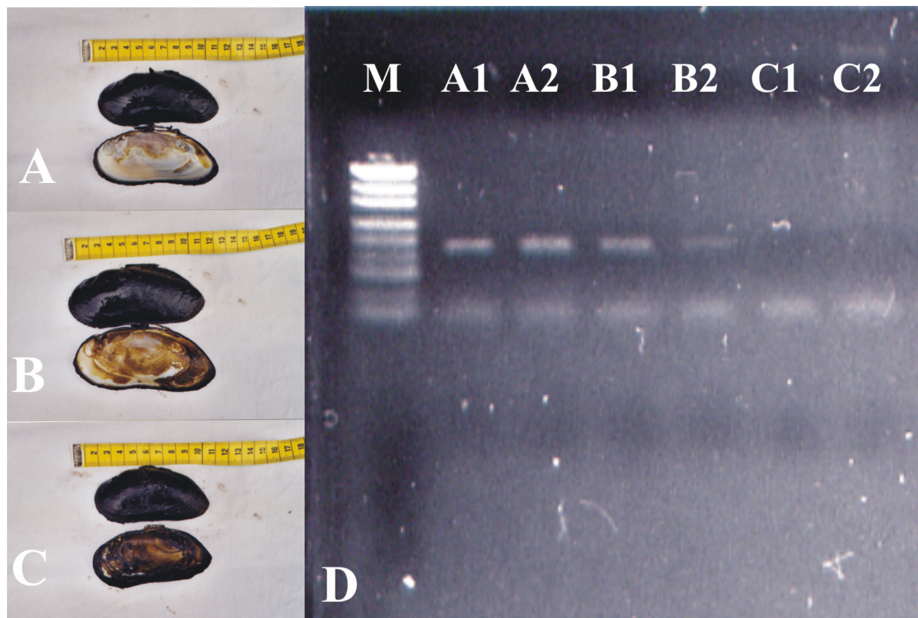


Fig. 5.1: **Freshwater pearl mussel (*Margaritifera margaritifera*) shells of different age and degradation (A, B, C) and electrophoresis of PCR-products of a species specific STR locus (D) with template DNA from theses shells; M= size marker.**

5.3 Applications of shell DNA analyses

Shell DNA analyses offer a series of potential applications. For instance, genetic parameters within populations (effective population sizes, genetic variability) and their history (pedigree-analyses, genetic drift and inbreeding) can be analysed from dead individuals and compared with actual parameters of their contemporary relatives. These data can reveal genetic changes, such as bottleneck effects, and thus provide insights into the life-history of specific populations. For restocking or reintroduction purposes, genetic information obtained from ancient shells may also be a valuable tool for selecting locally adapted populations from the same Evolutionary Significant Unit (ESU) or Conservation Unit (CU). Another application of shell material is to study genetic parameters between populations and to investigate past or present levels of gene flow, migration, hybridisation and genetic differentiation. Shell samples from extinct taxa or populations can contribute to a better understanding of evolutionary and phylogenetic processes linked with phylogeography between extant and extinct taxa or populations. Similar to forensic applications, shell DNA analyses may also be useful for species identification, e.g. in the context of the convention on international trade in endangered species of wild fauna and flora (CITES).

5.4 Factors influencing the success of shell DNA analyses

Several factors can influence the potential usefulness of mollusc shells for DNA-based analyses (Figure 5.2). The quantity and quality of extracted DNA from shell material largely depends on the mollusc species, its shell composition and shell crystal structure. Among freshwater bivalves, species with massive shells, a thick periostracum and a high organic content within the shell tend to yield comparatively high quantities of DNA. Physical and chemical degradation before sampling and during storage of shells can happen, especially if samples are exposed to extreme temperatures, UV light or to high humidity. Furthermore, the correct processing of shells and especially the grinding procedure is critical, with intense grinding and fine shell powders often reducing the usefulness of the sample, as degradation of DNA and increased binding of DNA to the matrix structure of fine shell powder can occur. For ensuring a uniform lysis of shell powder, the use of a shaking incubator is highly recommended. If standard phenol-chloroform DNA extraction procedures yield promising quality and quantity of DNA but no polymerase-chain-reaction (PCR) products are obtained, then prior removal of inhibitors with wash buffers on silica-based extraction procedures can be useful, similar to extraction procedures applied for aDNA.

A series of potential errors can happen when analysing samples with low quantities or poor quality of DNA (Figure 5.2), depending on the type of genetic analysis. Special concern must be attributed to avoid cross-contaminations with non-target-DNA between and within species. The use of universal primers, binding to conserved DNA-regions, can result in cross-species amplification even between not closely related taxa (e.g. bivalves – algae). Cross-species contaminations between molluscs have to be considered especially during processing of samples in the laboratory. Whereas all cross-species contaminations can be detected with species-specific markers, within-species contaminations are more difficult to detect and become critical when processing low amounts of target DNA. Genotyping errors can result in the missing detection of alleles (allelic dropout), the misinterpretation of lacking amplification of certain loci (false null alleles), or the incorrect detection of PCR-product lengths (false alleles / chimeric alleles; e.g. Taberlet *et al*., 1999). These effects are often connected with degradation and low concentration of template DNA. Sequencing errors can result from partial misincorporation of false nucleotides during PCR or jumping PCR, primarily caused by hydrolytic and oxidative damage of DNA, such as deamination of cytosine, depurination of adenine and guanine, oxidative dinucleotide modification or strand breaks (for review, see

Hofreiter *et al*. 2001a). Strongly degraded DNA is only suitable for amplification of short fragments. Amplification of mitochondrial genes tends to be more successful than PCRs with nuclear markers because of the large mtDNA copy number in each cell.

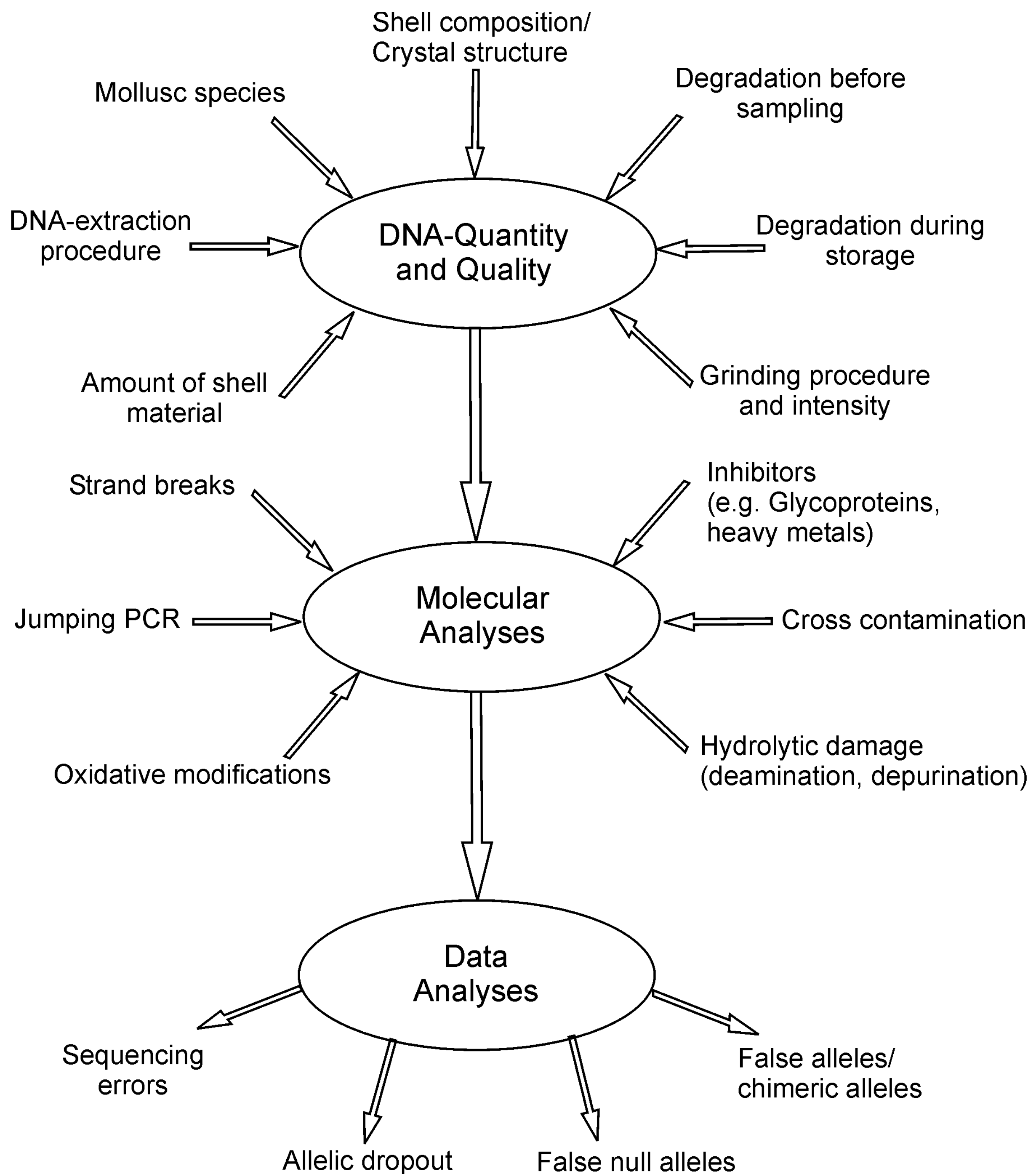


Fig. 5.2: Factors influencing quantity and quality of DNA and subsequent molecular analyses and their impacts on errors during data-analyses

5.5 Recommendations

Contaminations can be avoided by carrying out DNA extraction and PCR in different laboratories, by using filter tips for pipetting, by UV-treatment of buffers and equipment and by running negative controls during all steps of analyses. Before carrying out genetic studies based on shell samples of a certain species, it is necessary to initially evaluate the potential for erroneous results, to optimise an analysis strategy and to meet authenticity criteria to determine shell DNA (Table 5.1).

	Genotyping	**Sequencing**
Extraction repetition	Min. three independent DNA extractions per sample	Min. three independent DNA extractions per sample
Negative controls for extraction and PCR	Mock extractions and PCRs without template detect contamination during extraction and by PCR-buffers	Mock extractions and PCRs without template detect contamination during extraction and by PCR-buffers
Testing quality and quantity of DNA template	Pre-selection of suitable DNA using real-time PCR according to the minimum template amount needed by the selected marker-panel (see Morin *et al*., 2001)	Minimum number of DNA-molecules that initiate the PCR should be > 1000 revealed by real-time PCR (Hofreiter *et al*., 2001a)
Reduction of errors during molecular analyses	Reduced multiple tube approach (min. three independent repeats) according to Taberlet *et al*.(1999) in conjunction with the pre-selection method of Morin *et al*. (2001)	Independent PCR reactions and multiple cloning approach according to Hofreiter *et al*. (2001a,b)
Evaluation of data chromatogrammes	Exclusion of data under a certain minimum signal level and repeated data analyses by a second person	Comparison of sequence chromatogrammes (raw-data) of different independent multiple clones
Testing reliability in a second laboratory	Independent repetition of extractions, PCR-reaction and genotyping procedures for a few reference samples	Independent repetition of extractions; PCR-reaction, cloning and sequencing procedures for a few reference samples

Tab. 5.1: Authenticity criteria to determine shell DNA-data

Firstly, the sampling, the extraction procedures and the required DNA quality and quantity for the specific marker panel (dilution series PCRs), and the actual applicability of sampled DNA (Real-time PCR) should be tested. According to Hofreiter *et al*. (2001a) an experiment requires at least three independent repetitions if the number of template DNA molecules is less than 1000. Genotyping errors can be reduced by a multiple tube approach (Taberlet *et al*., 1999) in combination with the approach of pre-selecting samples with suitable template DNA by real-time PCR as described by Morin *et al*. (2001). This is an appropriate method to avoid erroneous results due to false or chimeric allele detection, allelic dropout and false null alleles. Semi-nested PCR (Bellemain & Taberlet, 2004) can be used when conventional PCR methods experience low success due to limited DNA concentration and/or quality. Sequencing errors can be minimised when carrying out sequencing from independent reactions and clones and by comparing those results with references. In order to reduce the number of templates with damaged bases, Uracil-N-Glycosylase treatment (Hofreiter *et al*., 2001b) can be applied and sequencing results before and after treatment can be compared. Such treatment, however, is expected to reduce the starting template copy number since UNG creates abasic sites, which rapidly result in strand breaks upon heating during PCR, thus limiting the number of samples suitable for study.

6 Stable carbon isotopes in freshwater mussel shells: Environmental record or marker for metabolic activity?

published: Juergen Geist, Karl Auerswald, Arnoud Boom (in press): Stable carbon isotopes in freshwater mussel shells: Environmental record or marker for metabolic activity?; *Geochimica et Cosmochimica Acta*

6.1 Abstract

Mussel shells have been used in a number of palaeoecological and environmental studies. The interpretation of stable carbon isotopic composition of shell material is still controversial. The carbon for shell carbonate precipitation can either be derived from ambient dissolved inorganic carbon (DIC), with shells recording environmental signals, or from metabolic CO_2, with the potential to disguise environmental signals. In order to gain insight into this question, we investigated four nearly 100-year long-term records of aragonite shells from an extant freshwater bivalve species, the endangered freshwater pearl mussel (*Margaritifera margaritifera* L.). Single growth increments of the outer prismatic and the inner nacreous zones were successfully and easily separated with a simple heat treatment for chronological analyses of $\delta^{13}C$ in single layers of each zone. Autocorrelation and semivariance statistical methods reveal that mussels show distinct individual signal patterns, which extend up to 25 years. Signal patterns are reliably reproduced with replicate samples from defined layers within one shell and show similar patterns with a slight offset for inner nacreous and outer prismatic layers for individual animals. Mussels exposed to the same environmental conditions exhibit distinct and contradictory signature patterns, which do not match between individuals. This observation can only be explained by strong metabolic influences on shell precipitation. Environmental changes in pH, temperature, electric conductivity and atmospheric carbon signature had no or little (<5%) influence, whereas body tissue protein and body tissue $\delta^{13}C$ signatures negatively correlated with the youngest produced shell $\delta^{13}C$ signatures, indicating that respiration causes a preferential loss of light isotopes from body mass and an inverse enrichment in shell aragonite. Hence, the shells of the freshwater pearl mussel yield a long-term record of metabolic activity, whereas the use of $\delta^{13}C$ in these shells as recorder for environmental signals is questionable. This may also be true for shells from other species, for which metabolic carbon incorporation has been acknowledged.

6.2 Introduction

Mussel shells have been used in a number of palaeoecological and environmental studies. While stable oxygen isotopic signatures in mussel shells have proved to be reliable recorders of environmental parameters (e.g. Epstein *et al*., 1953; Tripati *et al*., 2001), the interpretation of stable carbon isotopic composition of shell material remains contentious. Some isotopic studies on shells have shown that the stable isotopic composition ($\delta^{13}C$) of the shell carbonate is governed by the $\delta^{13}C$ of dissolved inorganic carbon (DIC) and therefore records changes in environmental variables such as pH, temperature, and salinity (e.g. Craig, 1953; Keith *et al*., 1964; Mook, 1971; Fritz & Poplawski, 1974; Donner & Nord, 1986). Under ideal conditions, shell carbonate would be precipitated in equilibrium, resulting in calcite which is +1‰ enriched in comparison with bicarbonate, and aragonite that is +2.7‰ enriched (Romanek *et al*., 1992). On the other hand, shell carbonates were often found not to reflect the predicted equilibrium fractionation, being in general less enriched than predicted in ^{13}C (e.g. Klein *et al*., 1996; McConnaughey *et al*., 1997; Kaandorp *et al*., 2003; McConnaughey, 2003). Most authors explain this offset by a contribution of metabolic carbon (Tanaka *et al*., 1986; Klein *et al*., 1996; Veinott & Cornett, 1998; Vander Putten *et al*., 2000). Thus, researchers have indirectly acknowledged an influence of the food source, which is reflected in a certain percentage of metabolic derived carbon within the carbonate. Despite this, it is often stated that the total contribution of metabolic CO_2 in aquatic invertebrates is generally low to insignificant, suggesting that kinetic effects can explain non-equilibrium fractionations (McConnaughey *et al*., 1997; McConnaughey, 2003). In contrast, Dettman *et al*. (1999) found $\delta^{13}C$ values of aragonite-forming freshwater bivalves with a highly variable offset to equilibrium values, suggesting a significant and variable incorporation of metabolic carbon into shell carbonate. Despite the observed isotopic disequilibrium of carbonate formation, shell records are frequently compared to or assumed to reflect changes in ambient $\delta^{13}C_{DIC}$.

The freshwater pearl mussel (*Margaritifera margaritifera* L.) is a long-lived species which is sessile or relative immobile during its adult phase. It produces a shell of well-defined material with annual increments. This combination offers a great potential for chronological analyses of shell $\delta^{13}C$ signatures to reveal changes within its environment. Freshwater pearl mussels are widely distributed in the holarctic range. They attain individual ages of more than 100 years (Bauer, 1992). The species is nowadays critically endangered and analyses of biochronological records in shell material may help to find reasons for the species´ low and

decreasing vitality during the last decades. Like other mussels from cold and temperate climates, *M. margaritifera* shells have annual growth increments (Figure 6.1), analogous to tree rings, with shell formation during summer and a reduced or ceased carbonate deposition at low temperatures during winter (e.g. Siegele *et al*., 2001). The inorganic carbon in *M. margaritifera* shells is carbonate in the form of aragonite (Carell *et al*., 1987; Nyström *et al*., 1995). Visible opaque, organic scleroprotein-rich ridges are produced during winter and separate the aragonite increments of maximum growth from summer seasons. These layers allow analyses of time scale series of samples. Once deposited, the carbonate is immobilized and resistant to subsequent changes (Lindh *et al*., 1988). *M. margaritifera* mussel shell records have been studied to reveal changes in elemental composition of the shell material and have been used as environmental indicators for eutrophication and acidification (e.g. Carell *et al*., 1987; Lindh *et al*., 1988; Mutvei & Westermark, 2001 and references therein).

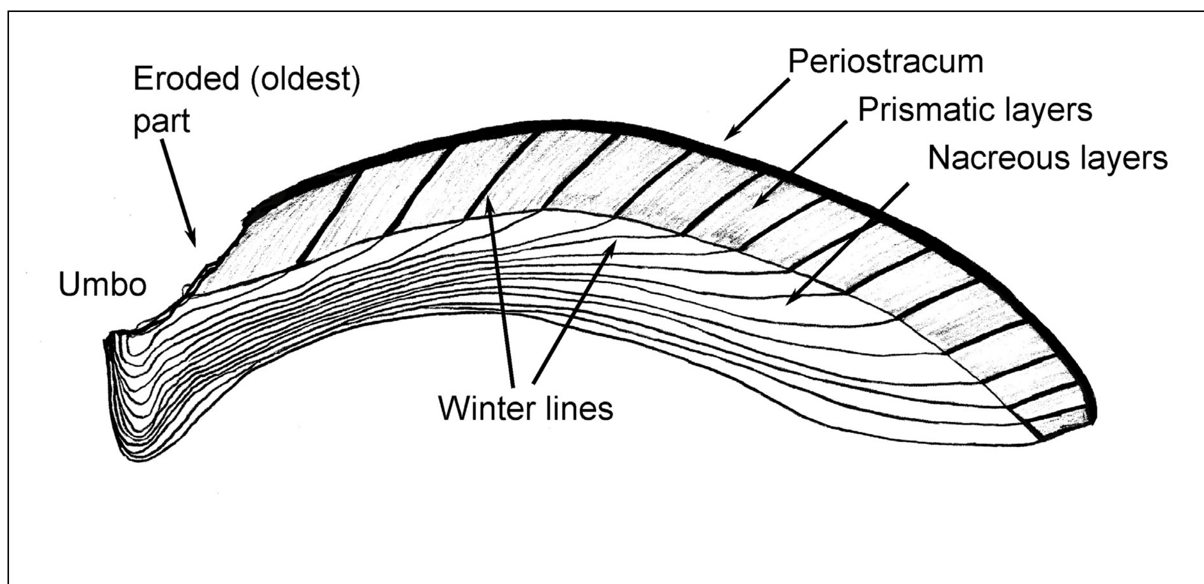


Fig. 6.1: Schematic of the cross-section of a *Margaritifera margaritifera* shell.

An evaluation of the influences on shell isotopic chemistry is important for the interpretation of such values in the context of environmental or palaeoenvironmental studies. We present a novel and simple method to sample annual growth layers from the outer prismatic and the inner nacreous zone of mussel shells, by removing the organic carbon, and we test the hypothesis that shell $\delta^{13}C$ signature is controlled by environmental variables. If this is the case, different individuals exposed to the same environment should exhibit similar $\delta^{13}C$

values and their signature should synchronously change with changing environment. Hence, we use old animals from one species and from the same location. Over their life span of one hundred years, changes in environmental conditions can be chronicled.

6.3 Material and Methods

Sampling site

Freshwater pearl mussels typically inhabit clear streams which are low in lime and nutrients. Four specimens originating from the same population were collected in August 2001 from a small headwater stream from the Elbe drainage system in the Fichtel Mountains close to the border between Germany and the Czech Republic. Specimens were labeled Z1, Z2, Z3 and Z12. All individuals were found in close vicinity, belonged to one large mussel bank covering an area of 5m by 1m and were exposed to identical environmental conditions. Daily observation of the stream and its strictly protected mussel population enabled us to sample four pearl mussels shortly after they had died. The mussels had shell lengths of 10.8 cm (Z1), 9.5 cm (Z2), 9.2 cm (Z3) and 11.0 cm (Z12). Exact sampling location will be provided on demand but is not published here, as illegal pearl fishing is still a threat to the endangered and protected species.

At the sampling site, the stream is on average 1.5 m wide and 25 cm deep. The stream is oligotrophic and the turbulent current results in a well-mixed water body. Oxygen concentrations and saturations measured in the field in 14-day intervals over three years showed an arithmetic mean of 11.1 mg/L (standard deviation, SD=1.4), equivalent to average saturation levels of 90% (SD=2.3). Biological oxygen demand over 5 days (BOD_5) was permanently low, with an average of only 1.1 mg/L (SD=0.5). Calcium concentrations averaged 7.0 mg/L with a standard deviation of 1.7 over the year. Data on pH, electrical conductivity and water temperature were available from 1987 to 2003 (provided by the Water Authority at Hof). Every year instantaneous measurements were taken during one day before the onset of the growing period (mostly in February), one day during the early growing period (mostly in April), one day in the middle of the growing period (mostly in August) and one day at the end of the growing period (mostly in October). Additionally, 5-day averages of air temperature measured at a meteorological station 30 km away from the brook were used to extend the temperature data set back to 1947 (Source: German Meteorological Service) as air and water temperature were closely linearly related (r^2=0.93 for n=67). The water temperature

ranged between 0 and 15°C and had a seasonal trend from about 2°C in January, a rise to 15°C in July and a decrease to 4°C in November. The pH mostly varied between 6 and 7, with snowmelt runoff early in the year causing the lowest pH of around 6.3. The average pH increased until the end of the year to 7.0. Low pH values (down to 5.5) caused by interflow runoff during heavy rainstorms are also possible throughout the year (Auerswald, 1990). No long-term trends in pH or temperature extending over several years were obvious from the data (Figure 6.2). Conductivity varied between 70 and 140 µs/cm, mostly with higher values during summer and lower values during winter and spring season, when water flow increases. Average values of temperature, pH and conductivity were calculated for the growth periods and compared with annual isotopic signatures, respectively.

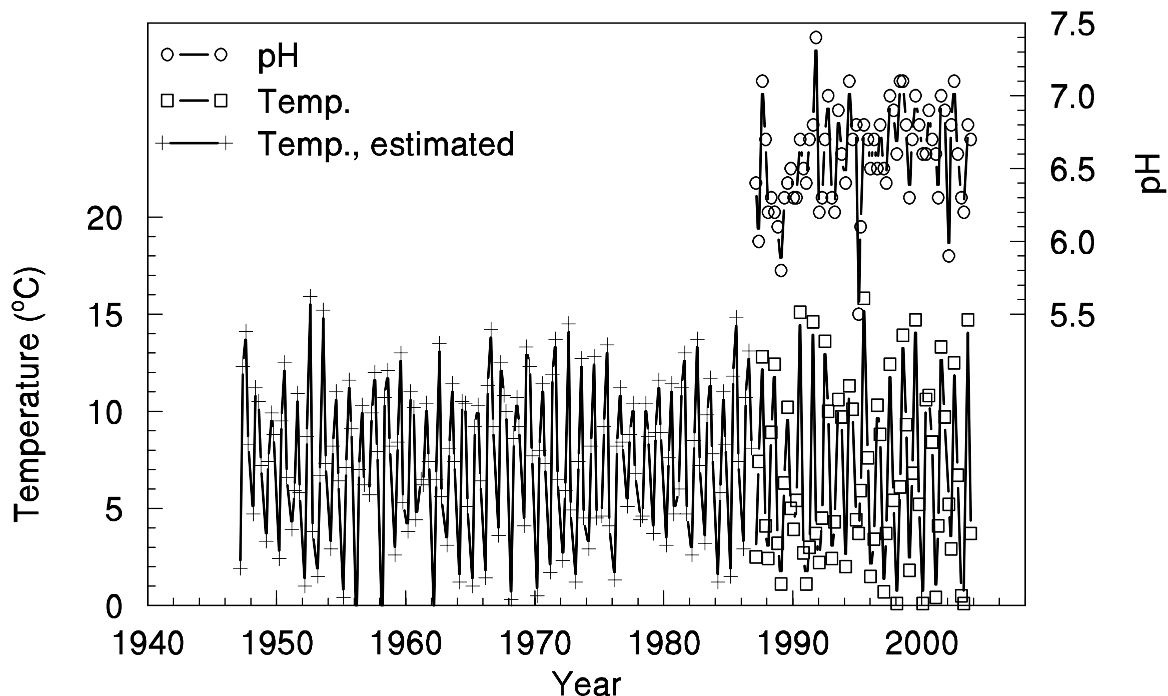


Fig. 6.2: Instantaneous measurements of pH and temperature from the brook water between 1987 and 2003. Water temperature was extended to 1947 by using daily averages of air temperature and a regression between air and water temperature.

Shell preparation

Margaritifera shells are composed of three principal zones: a moderately thick, organic periostracum on the outside of the shell preventing dissolution, followed by two aragonite containing zones, the outer prismatic and the inner nacreous layers (Figure 6.1). The oldest part of the shell is the umbo, where erosion of periostracum and aragonite occurs in older individuals.

Sampled mussels were preserved by freezing at -20°C. Soft tissue was manually removed and the shells cleaned and rinsed with H_2O (deionised) both in- and outside. For analyses of $\delta^{13}C$ of shell material, valves were separated by hand and from each shell, a 10 mm wide section was cut with a saw from the umbo region to the posterior-ventral edge, representing the axis of maximum growth and thus yielding the most detailed archival information (Siegele *et al.*, 2001). After drying at 50°C for 12 hours, shell sections were weighed and heated at 550°C for 2 hours in a muffle oven for complete combustion of organic components. After cooling down, the loss of weight on ignition was determined. The loss of organic material concentrated at the winter lines and allowed an easy separation of growth increments in both the outer prismatic zone and the inner nacreous zone, whereas the protein-rich periostracum simply fell apart and its remains were removed with a brush before sampling. The roofing-tile like layers of the outer prismatic zone were chronologically sampled with a scalpel blade starting close to the eroded part of the shell near the umbo towards the youngest layer at the ventral edge, which was produced in the final year of growth. The sampling of the inner nacreous layers was started from the inside of the shell towards the outside, representing a chronological order from the final year of growth towards older layers.

Growth layers in both zones, outer prismatic and inner nacreous usually correspond with years. However, growth interruptions during summer may result in overestimation of years and thin organic layers corresponding with short and warm winters can be overseen and thus two layers sampled as one. Both situations are rare and partly compensate each other in the long-term trend. Although visual inspection allowed an easy identification of single layers, it was occasionally not possible to separate them due to the high age (>80 yr) and slow growth of pearl mussels at the sampling site (maximum shell thickness before ignition: 3 mm; average layer thickness often around 0.7 mm for prismatic layers with a decreasing tendency towards ventral edges and more than ten times smaller for nacreous layers). In such cases, coherent layers were sampled together and the value was assigned to both layers. For one

shell (Z3) inner nacreous and outer prismatic layers were sampled from both valves independently in order to test the reliability of the method.

Stable carbon isotope analysis

Growth layers were ground to a fine powder with a carbon-free agate mortar and pestle. Three mg subsamples (+/- 0.1mg) were then enclosed in tin cups (4x6mm) and combusted in an elemental analyser (Carlo Erba NA 1108, Milan), interfaced (ConFlo II, Finnigan MAT, Bremen) to an isotope ratio mass spectrometer (Delta Plus, Finnigan MAT, Bremen). Different tissues of the soft parts of the mussels (mantle margin, mantle interior, mantle total, adductor muscle, gills, foot and digestive gland/visceral mass) and samples of potential food sources (alder leaves, roots from ambient riparian vegetation, coarse detritus, fine detritus and fine suspended particulates >30 µm) were freeze-dried, ground and ball milled before analyses. Organic shell material was analysed after ball-milling shell material and subsequent HCl fumigation of the moistened sample material, previously weighed into Ag cups (Harris *et al*., 2001). Nitrogen signatures were directly measured from ball-milled untreated samples. Proteins and lipids were separately extracted from the body soft tissue of the four specimens and analysed for $\delta^{13}C$ and $\delta^{15}N$, according to Rossmann (2001) and Piasentier *et al*. (2003).

The data are presented as $\delta^{13}C$ (‰) relative to PDB standard. For possible food sources and tissue material $\delta^{15}N$ (‰) was additionally measured in relation to nitrogen in air. $\delta^{13}C$ and $\delta^{15}N$ were calculated as follows: $\delta X = [(R_{sample}/R_{standard})-1]*10^3$, where δX is $\delta^{13}C$ or $\delta^{15}N$, and R is the respective $^{13}C/^{12}C$ or $^{15}N/^{14}N$ ratio. The working gas standards for C and N isotope determination were calibrated against the laboratory standard, a fine ground wheat flour of known C and N isotope composition ($\delta^{13}C$ -26.54‰ and $\delta^{15}N$ +2.61‰), which had previously been calibrated against IAEA-CH6 and IAEA-NO3 secondary standards. The same working standard was run regularly after every 10th sample as a control. Blank determinations were done routinely before each batch of samples (including working standards) by running empty tin cups. The $\delta^{15}N$ data were blank-corrected. The external precision (standard deviation, SD) was ±0.2‰ for $\delta^{13}C$ and ±0.3‰ for $\delta^{15}N$.

A series of additional tests were carried out in order to verify the impact of the shell analyses procedures in comparison with conventional methods. X-ray diffraction analysis was used to investigate conversion of aragonite to calcite. Removal effectiveness of organic components during heating was verified by two independent experiments: firstly, removal of N in the shell

samples was measured by combustion with the elemental analyzer. Additionally, carbon removal efficiency was investigated at various temperatures, using a mixture of silicate powder and wheat flour. Accuracy of the measurements in comparison with standard phosphoric acid digestion technique using Kiel device was tested by splitting samples and analysing $\delta^{13}C$ signatures of powdered shell material (each 15 samples before and after ignition at 550°C) in two laboratories.

Statistical analysis

Autocorrelation analysis and geostatistical analysis (semivariograms) were applied in order to detect and determine the extent of layer overlapping signal trends in $\delta^{13}C$. Experimental semivariograms for $\delta^{13}C$ were computed to determine the extent and range of autocorrelation by pooling the inner and outer layers of all shells. Semivariograms quantify the average dissimilarity (=semivariance) of a property (e.g. the signature) depending on the distance (=lag), which is in this case the number of layers between two samples. Spherical models were fitted to the experimental semivariograms. The intercept on the y-axis (semivariance for zero lag) is called the nugget effect and quantifies local variability or “noise” within the data. With increasing lag, semivariance approaches a plateau, which is called the sill. The sill of the semivariogram quantifies the variation in signature over distances beyond the range of the autocorrelation. A large difference between sill and nugget effect indicates a pronounced pattern, while no pattern exists where the sill equals the nugget effect. The range corresponds to the lag beyond which the sill is reached. It quantifies the maximum distance over which pairs of observations remain correlated. For theory and details of geostatistical analysis see Nielsen & Wendroth (2003). Most statistical calculations were done with SAS (version 8; SAS Institute, Cary, USA).

6.4 Results and Discussion

Sampling method and reliability

Heating at 550°C indicated 7-8% (wt.) organic material (av.=7.6%, SD=0.7%) and was an easy and effective method to remove the periostracum, separate the outer prismatic and inner nacreous layers and sample distinct growth increments within these zones. More than 60 layers per shell could be separated. This method allowed an easy sampling of the roofing-tile like prismatic (=outer) layers. Single “roofing tiles” from the outer prismatic zone consisted

of one or two growth increments in 90% of all cases, whereas separation was less effective for the thinner inner nacreous layers where the samples mostly consisted of two growth increments (Figure 6.3). For the two replicates of mussel Z3, similar numbers of years were sampled (83 vs. 86 years in outer layers and 49 vs. 47 years in inner layers).

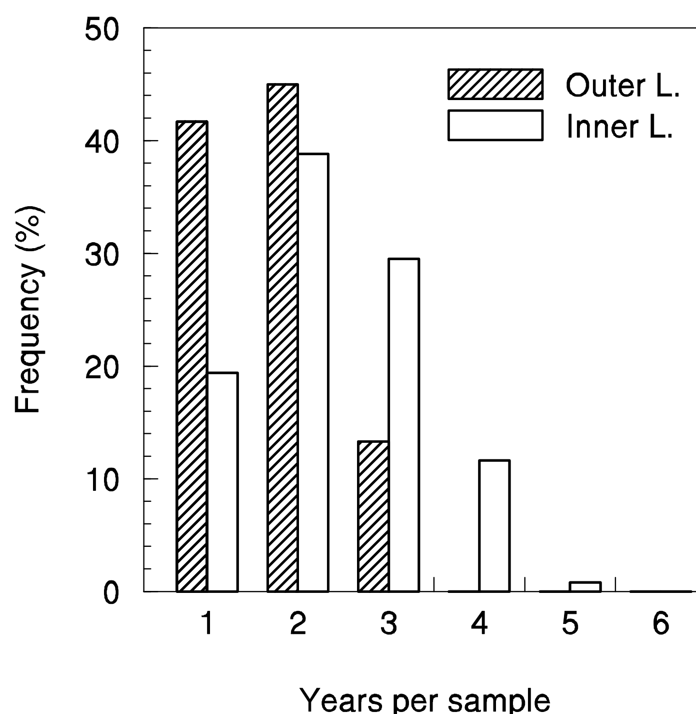


Fig. 6.3: Number of years per sample of the outer and inner layers as estimated from visual inspection.

While the outer layers are preferable to study inter-annual variations, it has to be considered that erosion occurs at the umbo region of old shells in freshwater pearl mussels and some other mussel species. This will reduce the time span of the archive. Nevertheless, for mussel Z3 more outer layers than inner layers were found, which may depend on the specific erosion pattern in this particular shell.

Other sampling techniques for shell material, such as micro drilling and milling of shell material at depth intervals of 15-30 µm (Dettman & Lohmann, 1993; Dettman *et al*., 1999; Wurster *et al*., 1999), are able to deliver a higher time resolution than the method described in this paper and even allow studies on intra-annual variation. Such techniques, however, require specific equipment and sampling may be restricted to certain areas of the shell because of geometry requirements of the sampling technique (Dettman *et al*., 1999). Furthermore, drilling techniques likely increase the danger of yielding mixtures of nacreous and prismatic

layers or different annual layers within these zones, which may especially arise when mussels with thin shells, high individual age and moderate growth rates are selected for investigation. For studies on long-term trends and comparisons of average values between growth increments (usually corresponding with years in cold and temperate climate zones), the methodology suggested in this paper allows an easy and reliable sampling of defined layers for both, the prismatic and inner nacreous zone, separately. However, each sampling technique is based on the assumption that the annual growth pattern in the shell with organic-rich layers and ceasing carbonate precipitation at lower temperatures during winter prevails, and is susceptible to counting errors. For *M. margaritifera* these errors are considered to be small, and the tree ring analogous shell growth patterns are regularly used by field biologists for individual age determination in the species. The difference of growth layers sampled in both valves of Z3 was 3% for the outer prismatic and 4% for the inner nacreous layers, respectively, which is in good agreement with an estimated counting error of +/- 5 percent in 100 years for *M. margaritifera* reported by Carell *et al*. (1987). As freshwater pearl mussels used in this study were comparatively old and as the species is reported to grow in approximately asymptotic fashion (Hastie *et al*., 2000), the use of younger and faster growing individuals from this species or the use of generally faster growing mussel species will probably allow further reduction of this error. For analyses of time trends, small errors in layer sampling will be of minor importance. In cases where more exact layer sampling is required, the analyses of several independently sampled stripes from both valves of one shell and subsequent comparisons of time trends will improve dating.

A series of additional tests (XRD-analysis, comparison of the method with standard phosphoric acid digestion technique, C and N-measurements for testing complete combustion of organic matter in the shell material during heating) proved the reliability of the methods described in this paper. Aragonite was completely converted to calcite but the procedure proved not to systematically change the $\delta^{13}C$ signatures (average difference between heated and untreated samples determined by standard phosphoric acid digestion: -0.2‰$^{n.s.}$). The only effect of the heating was an increase in scatter. Therefore, the identified patterns in subsequent layers may in fact be even more pronounced. The ignition process at 550°C removes all the organic carbon and 97% of the nitrogen and was found to be less effective at lower temperatures. Assuming a worst case situation, the complete conversion of all potentially remaining organic carbon to carbonate during the heating (assuming a $\delta^{13}C$ signature of -27‰), the $\delta^{13}C$ signature of shell samples could only be shifted by a maximum

of 0.5‰. Even these worst case assumptions would thus neither change the ranking of samples nor limit the comparability of patterns.

Mussels display long-term $\delta^{13}C$ patterns

Values for $\delta^{13}C$ in *M. margaritifera* shells ranged from -10‰ to -15‰. Similar values were described for *Elliptio complanata*, another freshwater mussel species, where maximum range of $\delta^{13}C$ variation was -9.0‰ to -14.5‰ and annual variations of maximum 2‰ (Veinott & Cornett, 1998). In generally faster growing zebra mussels, Fry & Allen (2003) found less negative carbon isotopic signatures, varying between -8‰ to -11‰ in one river but to be quite constant around -9‰ at one location, with slight seasonal variation of less than 0.5‰.

Autocorrelation analyses showed that $\delta^{13}C$ signatures were similar in adjacent growth layers, but dissimilarity increases with distance. Signatures of the youngest layer (2001) from different mussels closely correlated (r^2=0.96 with n=5, outer prismatic layers) with the previous layers of the same mussel (Figure 6.4A). With increasing distance to the latest produced layer, correlation decreased sharply. This indicated that (i) mussels show distinct individual signatures differing by 3‰, although they grew in close vicinity, and (ii) that these patterns cover several years.

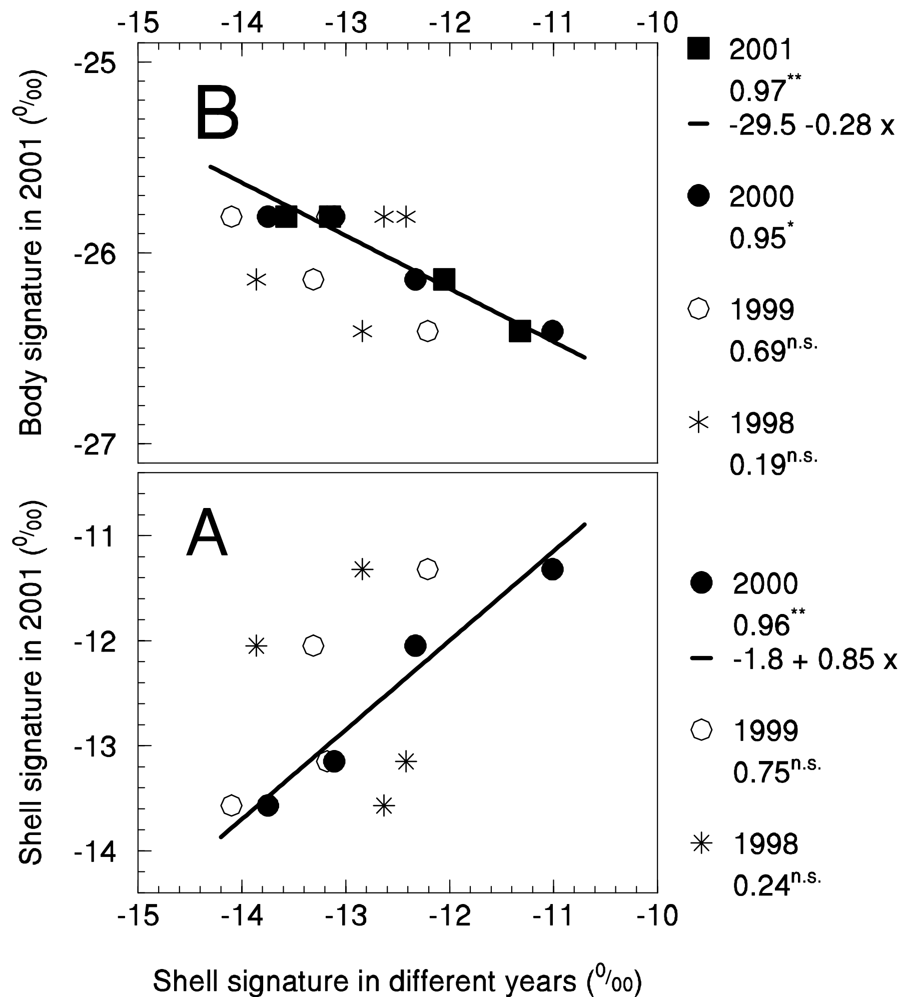


Fig. 6.4: Correlation of the $\delta^{13}C$ signature of shell carbonate from the last (1998-2000) outer layers with the youngest (2001) shell carbonate (A) and with the organic carbon in body tissue (B).

Geostatistical analysis allowed a more rigid assessment of this phenomenon by taking all layers of all mussels simultaneously into account. This also showed that adjacent layers were autocorrelated and did not show independent signals. The range shows that the autocorrelation extended up to 25 layers (Figure 6.5, Table 6.1). The resulting temporal pattern (sill) contributed about 70% to the total variation (sill+nugget), while the nugget effect, which quantifies the variability within a certain layer, contributed the remaining 30%. Hence, 70% of the signal can be interpreted as a non-random time trend and 30% of the signal is a layer-individual (= annual) signal plus the analytical error.

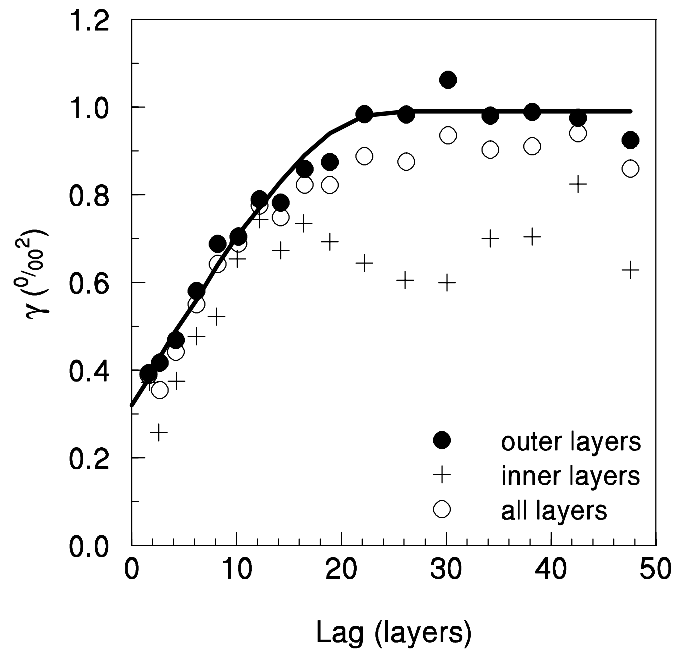


Fig. 6.5: Pooled semivariograms of $\delta^{13}C$ signature for the inner, outer and all layers; parameters of the spherical model calculated for the outer layers (line) are given in Table 6.1.

	outer	inner	all	unit
Nugget effect	0.32	0.15	0.25	‰²
Sill	0.67	0.55	0.65	‰²
Range	25	15	21	years
RMSE	0.035	0.063	0.035	‰²

Tab. 6.1: Parameters of spherical models fitted to the experimental semivariograms of the outer and inner layers (RMSE is the Root Mean Squared Error between the semivariogram model and the experimental semivariogram).

The nugget effect, sill and range were smaller for the inner nacreous layers than for the outer prismatic layers. All three effects resulted from the higher proportion of composite samples of the thin nacreous layers.

Carbon sources for aragonite formation

Industrialisation linked with burning of fossil fuels has changed atmospheric CO_2 signature during the last decades towards more negative $\delta^{13}C$ values. This atmospheric trend should have caused an analogous increase in the part of the DIC, which may be directly derived from the atmospheric pool and in the signature of new primary biomass, which is a potential food source for freshwater pearl mussels. Chronological samples of shell material should reflect this atmospheric trend if either (i) DIC governed by atmospheric CO_2 signature or (ii) a high percentage of C from metabolised fresh primary biomass is incorporated during shell aragonite precipitation. While the overall trend in the shells was -0.0039‰ yr^{-1}, the global atmospheric trend was much stronger with about -0.0094‰ yr^{-1} between 1990 and 2000 and -0.0295‰ yr^{-1} on average after 1960 (Figure 6.6). Even if we consider that the large scatter in shell data due to physiological influences will decrease the slope of the regression for the shell, the pronounced trend in air signature after 1960 was not reflected in the mussel shells. We can exclude that temperature effects have disguised the atmospheric trend. The equilibrium ^{13}C fractionation during aragonite precipitation relative to CO_2 was determined by Romanek *et al*. (1992) $\varepsilon_{aragonite\text{-}CO2} = 13.88 - 0.13$ (t°C). From this temperature-fractionation relationship an increase in water temperature by 10.6°C would be necessary to compensate the atmospheric change in carbon signature of 1.245‰ over 50 years. Such an increase in water temperature is highly unlikely and can be excluded when considering air temperature trends from the region (Figure 6.2). Hence, the independence of shell signal from atmospheric trends can only be explained by the incorporation of old (before 1960 or even pre-industrial) or mixed organic carbon of different age into the shell.

Unionid bivalves are semi-infaunal filter-feeders and their primary food is believed to be fine particulate organic matter suspended in river water (Dettman *et al*., 1999). However, the food source of freshwater pearl mussels is not known in detail and is still subject to speculation. The internal productivity of this stream is low due to the low nutrient level and shading by trees. The main food source therefore probably originates from the terrestrial surroundings. Isotope analysis of possible food sources showed a more or less uniform $\delta^{13}C$ around -28.2‰ with the exception of fine suspended particulates, which are only -27.0‰ (Table 6.2). Differences in signatures are even larger for $\delta^{15}N$, which ranged from zero to -2.5‰ for all materials except for the fine suspended particulates, which were 5.0‰, and which were two

trophic levels above other sources if we assume a trophic shift of 3‰ per level (De Niro & Epstein, 1981; Ponsard & Averbuch, 1999).

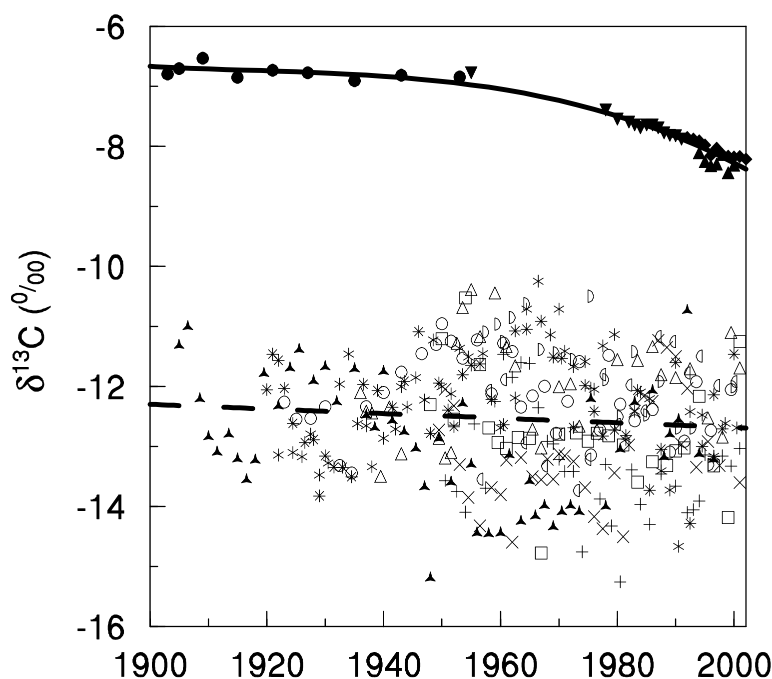


Fig. 6.6: Comparison of shell signature (crosses for outer layers; open symbols for inner layers) with atmospheric CO_2 signature (filled symbols) reconstructed from annual averages from the Siple icecore (Antarctica; Friedli *et al.*, 1986), and the atmospheric measuring stations of Mauna Loa (Hawaii, Keeling *et al.*, 1995), Hungary, Ulan Uul (Mongolia) and Ochsenkopf (Germany, http://www.cmdl.noaa.gov/ccgg/iadv/); regression lines: air with $r^2=0.9787$ for n=42, shells with $r^2=0.0083$ for n=345.

Potential Source	C content %	C/N	$\delta^{15}N$ ‰	$\delta^{13}C$ ‰
Decomposing alder leaves	42.1	24.4	-2.50	-28.03
Roots	41.3	28.5	0.60	-28.37
Coarse detritus	25.7	23.6	-0.68	-28.02
Fine detritus	10.6	13.4	-0.76	-28.23
Fine suspended particulates > 30 µm	22.0	12.9	5.00	-26.96

Tab. 6.2: Average C content, C to N ratio and C and N signatures of potential food sources (concentration of fine suspended particulates in brook water ~50 µg L^{-1}).

For molluscs, the trophic level shift is only 1 to 2‰, presumably because they excrete ammonium instead of urea or uric acid (Vanderklift & Ponsard, 2003). This is in agreement with the comparison between visceral mass, which consisted mainly of partly digested food, and the remaining body mass (Table 6.3). The trophic level shift for C in general is small, around 1‰ (De Niro & Epstein, 1981), which is also reflected by the difference between visceral mass and remaining body mass. Hence, the most likely food source should have had a $\delta^{15}N$ of around 5‰ and a $\delta^{13}C$ around -27‰. Both conditions were only met by the fine suspended particulates, which most likely contribute the main share to the mussel diet. The high ^{15}N value in this material in comparison with primary plant biomass indicates that it has passed considerable degradation and recycling. Hence, it is old and composite material of different age. In such material the atmospheric signal should be weaker than in air or primary biomass.

	n	$\delta^{15}N$	SE	$\delta^{13}C$	SE
Overall average	31	6.90	0.26	-26.10	0.16
Shell organic matter	16	6.81	0.41	-26.15	0.31
Body without visceral mass	13	7.27	0.95	-26.02	0.47
Visceral mass	2	5.80	1.84	-26.29	1.66

Tab. 6.3: Average $\delta^{13}C$ and $\delta^{15}N$ signatures of mussel tissues.

In principle, the carbon may stem from mussel respiration of organic material (food) or from riverine DIC, which is influenced by atmospheric CO_2 and environmental respiration (community respiration and input of runoff, soil water and groundwater carrying DIC derived from old plant matter). Assuming an air $\delta^{13}C$ signature of -7.8‰, and a fractionation of aragonite formation relative to CO_2 according to Romanek *et al*. (1992), then at a water temperature of 10°C a $\delta^{13}C$ value of +4.2‰ would be expected for aragonite formed at isotopic equilibrium. This differs considerably from the values measured in the shells. Analogously, we can assume a food signature of –27‰. A calculation of food consumption and respiration of the mussels indicates that for shell formation (on average: 0.09 g yr^{-1} $mussel^{-1}$), less than 10% of the respired CO_2 is needed, which allows discrimination to take place during aragonite formation. If this metabolically derived carbon is converted to

aragonite, applying the fractionation factor as mentioned above, at 10°C a $\delta^{13}C$ value of - 15.2‰ would be expected.

The shell signature and its variation can thus be explained by a varying contribution of air and respiratory C (mussel respiration or community respiration contributing to DIC) to the aragonite formation, with respiratory C contributing the largest share. The high contribution of respiratory CO_2, the variation among mussels and the weak to missing correlation with water properties over years are concordant with our perception that the variation in shell signature is of metabolic origin. A calculation of dissolved gasses based on Henry's law and water properties (temperature, pH, ionic strength) indicates that about reasonable 10% of the oxygen dissolved in the inhaled water has to be converted to CO_2 to balance the dissolved carbon species (CO_2, HCO_3^-, CO_3^{2-}) and thus can create a 50% metabolic signal in aragonite precipitated from the exhaled water. The lack of knowledge about the physiology and metabolic activity of freshwater pearl mussels, however, allows no interpretation of the correspondence between differences in signatures and physiological states. Investigations of the signature of recently precipitated shell material may allow to identify organisms in different physiological state and thus to gain better insight into this organism.

Mussels show individual signals

Both shell valves from Z3 showed a clear similarity (Figure 6.7), indicating the reliability of the analyses. The variance between the outer layers of the two valves (0.44‰²) was close to the nugget effect (0.36‰²). The nugget effect is estimated from the variation between adjacent layers of one valve and therefore independent from the correspondence of absolute years, to which the layers of both valves are assigned. The similarity of both values indicated that only little additional error results from assigning the layers to years. The same conclusion can be drawn from the comparison between the prismatic and nacreous layers of individual mussels (Figure 6.8), which exhibited similar patterns, although an off-set between prismatic and nacreous layers extending over several years was often observed.

While the patterns of the prismatic and nacreous layers of one individual were similar, those of different individuals did not match, even assuming errors in assignment to the time axis of single growth increments. This is a strong indication that physiological processes exert predominant influence on shell $\delta^{13}C$ patterns. This interpretation is supported by the strong

negative correlation of body tissue signatures with signatures of the youngest shell layers (Figure 6.4). Additionally, total protein mass in the soft tissue negatively correlated with body signature (r^2=0.76). The body mass varied by a factor of 3 (containing 0.5 – 1.5 g dry matter of protein) despite the similar and high age of the mussels. The studied specimens covered the whole mass range observed in freshwater pearl mussels at this site (Schreckenbach, 1995). This clearly indicates that they differed in metabolic state. The strong correlation with signature suggests that low-weight mussels (e.g. due to starvation or high metabolic activity and production of glochidia) preferentially respire the isotopically lighter amino acids, resulting in a lower body mass, which is isotopically enriched in ^{13}C. The increased use of isotopically lighter lipids (average difference to protein in our samples: -2.8‰) falls short, as their total amount is too low (<1% of body dry matter in all cases) in order to explain the observed pattern. Indeed, individual differences in the gross energy and soft tissue composition of female mussels from the same river were previously described (Schreckenbach, 1995). This study also revealed losses of 50% in gross energy and strong reduction of dry mass for *Anodonta anatina* mussels kept in tanks for 6 months without feeding. The strong correlation between body protein δ^{13}C signature and total body signature of mussels can be explained by the fact that 60-70% of the total dry mass of mussel tissue is made up by the raw protein fraction (Schreckenbach, 1995).

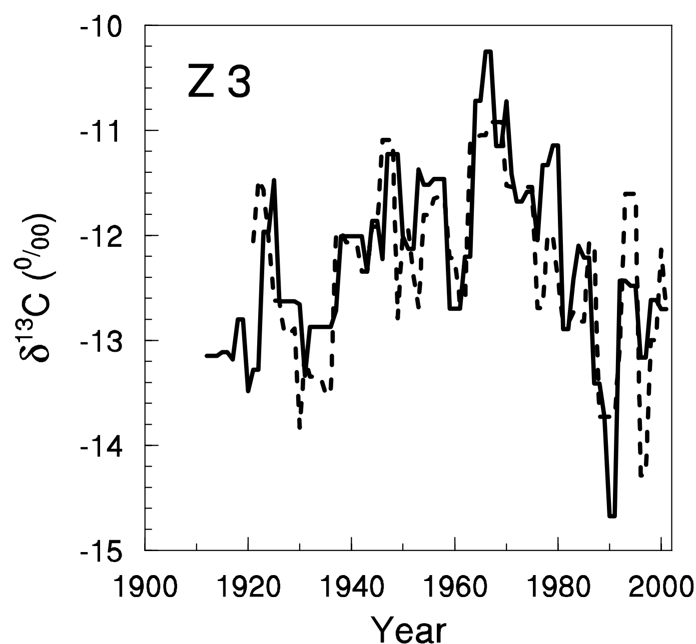


Fig. 6.7: Variation of δ^{13}C between successive prismatic layers of both valves from mussel Z3.

Indeed, environmental parameters averaged over the growth period explained very little to none of the variation in annual signature with r^2 being 0.047, 0.001, 0.0006 and 0.0005 for temperature, pH, conductivity and summer rainfall, respectively. Despite this low predictive ability, the correlation to temperature was highly significant due to the large number of samples (n=150 in each case). The slope was positive although fractionation decreases with temperature (Romanek *et al*., 1992). This indicates that the $\delta^{13}C$ of carbon source increased during the warmer months of the year, e.g. by increasing contribution of metabolic versus water derived CO_2. From the geostatistical analysis it followed that the periods of different metabolic activity extended up to 25 years. Factors like individual age, gender-associated differences, fecundity and filtering activity are related to metabolic activity and may result in varying body composition and shell carbon signatures between individuals exposed to the same environment. Dettman *et al*. (1999) found a 4 to 5‰ increase in shell $\delta^{13}C$ in North American freshwater mussels in early August when changes in temperature and $\delta^{13}C$ of DIC were minimal. They state that this change may be associated with the hatching and brooding of young in the marsupia. Metabolic differences between male and female mussels together with the highly variable fecundity among female pearl mussels (Bauer, 1998) could also explain the differences between the individuals investigated in this study. With some annual differences, *M. margaritifera* broods from July to September in the population investigated, which coincides with the period of maximum shell growth. Hence, annual growth increments might be expected to reflect such gender- or fecundity-linked physiological differences. Individual metabolic activity of pearl mussels may also be linked with genetic factors. Such investigations require high resolution genetic markers, such as microsatellites, which were recently established for the pearl mussel (Geist *et al*., 2003). For the marine mussel *Mytilus trossulus* Klein *et al*. (1996) showed that shell $\delta^{13}C$ was influenced by the rate of mantle metabolic activity and Wefer & Berger (1991) reviewed several studies and stated that $\delta^{13}C$ was recording metabolic activity and reproductive activity, which agrees with our results for the freshwater pearl mussel.

Differing filtering activities and food uptake could also be possible explanations for differences between individuals. Borchardt (1985) found an exponential increase of carbon incorporation efficiency and approximately linear increasing net corporation efficiency with decreasing food rations in the blue mussel *Mytilus edulis*.

Kinetic isotope fractionation effects during carbonate precipitation appear to be more likely in aragonites than in calcites and are expressed when calcification occurs within thin, alkaline, Ca^{2+} rich solutions separated from adjacent cells by CO_2 permeable membranes (McConnaughey *et al*., 1997). These effects could, in theory, cause considerable variation in shell $\delta^{13}C$, however, this would only occur during rapid carbonate precipitation and thus could only be expected during rapid skeletogenesis (McConnaughey, 1989). Therefore, kinetic isotope effects are highly unlikely to play a role in shell formation for these slowly growing pearl mussels.

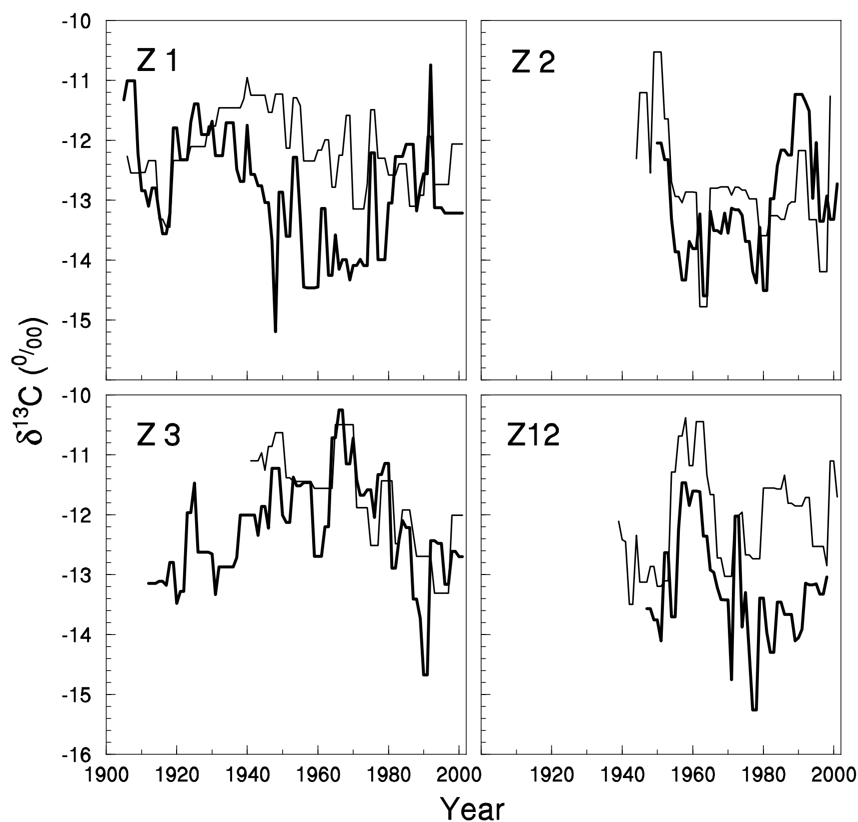


Fig. 6.8: Comparison of $\delta^{13}C$ patterns from all four mussels; thick lines are outer prismatic layers, thin lines are inner nacreous layers.

Theoretically, the differences between individual signals could also be explained by the exposure of individuals to different environmental conditions, resulting from (i) mussel migration or translocation from other sites or (ii) the existence of different microhabitats within the brook. As the investigated population has been well monitored, migration or translocation events can be excluded for at least 15 years and especially for the last four years before sampling. Different microhabitats, e.g. different pH linked with algae or submerse vegetation, cannot be fully excluded for the past, but flow turbulences result in a well-mixed

water body. Even assuming the occurrence of different sediment microhabitats in this stream with differing CO_2/O_2 ratios, the mussels would still all be exposed to the same water since adult pearl mussels do not bury themselves into the sediments at this site but inhale and infiltrate water from the free-flowing, well-mixed and well-aerated water body. Homogenous water chemical conditions around the mussel bank were also supported by spatially resolved (< 1m) measurements of temperature, conductivity and pH, which never showed significant differences in the flowing water body during measurements over several years.

6.5 Conclusions

The shells of freshwater pearl mussels provide a long-term stable carbon isotope archive, extending up to 100 years for central European populations. By heating at 550°C this archive can be easily separated into individual layers, which can then be assigned to single years. This separation is easier for the thick outer prismatic layers than for the thin inner nacreous layers. The outer layers are preferable for sampling in this respect, although some information from early growth stages may be lost by erosion of the oldest layers.

Following the findings of previous authors, shell carbonate is derived from ambient DIC and metabolic derived CO_2. The shells exhibit distinct patterns, which are for each individual similar for both valves and also for the inner nacreous and the outer prismatic layers. Different individuals, which lived within 5 meters of each other, and have been exposed to identical environmental conditions, have very different $\delta^{13}C$ patterns in their shells and soft tissues. The negative correlation of soft tissue protein mass with body signature suggests the preferential consumption of isotopically lighter amino acids in periods of starvation or increased metabolic activity, in turn resulting in a totally lighter but isotopically enriched body. The incorporation of the respired carbon in the aragonite results in a negative correlation between $\delta^{13}C$ signatures of the soft tissues with the youngest shell layers. Additionally, almost no influence of atmospheric CO_2 signature, pH and water temperature on $\delta^{13}C$ patterns was detectable. Consequently, the observed distinct signature patterns of different mussels can not be explained by environmental variables, but metabolic processes must exert predominant influence on shell signatures. Individual signal trends extend up to 25 years and can cover one fourth of the life span of the mussel.

Shell aragonite carbon mainly originated from respiration. The lack of an atmospheric trend in the $\delta^{13}C$ of the shell carbonates suggests that the respired carbon source consists of old recycled carbon. This is also in agreement with the $\delta^{15}N$ signatures of the body tissue, which showed a high trophic level. Both, the $\delta^{13}C$ and the $\delta^{15}N$ signatures, indicated that fine suspended particulates (>30 µm) are the most likely food source.

Our results indicate that $\delta^{13}C$ signatures in freshwater mussel shells can strongly be influenced by individual metabolic signals, which prevent these time archives from being used for reconstruction of environmental parameters. This phenomenon should also be taken into account when investigating other mussel species.

7 The status of host fish populations and fish species richness in European freshwater pearl mussel (*Margaritifera margaritifera*) streams

published: Juergen Geist, Markku Porkka, Ralph Kuehn (accepted): The status of host fish populations and fish species richness in European freshwater pearl mussel (*Margaritifera margaritifera*) streams; *Aquatic Conservation: Marine and Freshwater Ecosystems*

7.1 Abstract

The status of host fish populations and fish species richness was investigated at 36 sites of 20 extant freshwater pearl mussel populations, including the drainages of Elbe, Danube, Rhine, Weser, Aulne, Kemijoki and Tuuloma in the countries of Germany, the Czech Republic, France and Finland by carrying out comparative electrofishings.

Brown trout (*Salmo trutta* f. *fario*) were found to be the available host fish for pearl mussels in all except one of the investigated streams with mean densities of 2861 ha^{-1} (range 0-8710 ha^{-1}) and a mean biomass of 119 kg ha^{-1} (range 0-478 kg ha^{-1}). Streams that had been frequently stocked with brown trout had higher trout biomass and densities of host fish than natural populations, but trout stocking had no positive effect in two of the investigated streams.

Fish species richness ranged between two and 16 species per stream and showed a negative correlation with host fish biomass and host fish densities. Undisturbed oligotrophic pearl mussel headwater streams usually only yielded a low number of fish species. Habitat degradation can reduce competitiveness of specialised trout and result in an increased abundance of ubiquitous or atypical species.

A link between the lack of juvenile pearl mussels and a lack of suitable host fish was only rarely observed. Functional pearl mussel populations with relatively high numbers of juveniles had significantly lower densities and biomass of host fish than pearl mussel populations without recent recruitment.

This study suggests that 0+ host fish are not necessarily required to sustain functional pearl mussel populations. Low densities of host fish can be compensated by the higher glochidia carrying capacity of older host fish with limited previous contact to pearl mussel glochidia, by a long reproductive period of mussels, and by low mortality rates of juvenile mussels during their post-parasitical phase.

7.2 Introduction

The freshwater pearl mussel (*Margaritifera margaritifera* L.) was a formerly widespread and abundant species distributed from the Arctic and temperate regions of western Russia through Europe to the northeastern seaboard of North America. Several studies have revealed dramatic declines throughout its Holarctic range (e.g. Bauer, 1988), and the species is presently under a serious threat of extinction (Ziuganov *et al*., 1994, Young *et al*., 2001a). Few populations remain functional and still have a significant number of juveniles present (Cosgrove *et al*., 2000; Young *et al*., 2001a, b). To help establish the reasons for this lack of recruitment and the severe population declines, a sustainable conservation approach which integrates all critical stages in the complex life cycle of freshwater pearl mussels is required.

The slow-growing *M. margaritifera* is one of the longest-lived invertebrates known, capable of reaching ages up to 200 years (Mutvei & Westermark, 2001). In common with other freshwater bivalves, the sexes of *M. margaritifera* are usually separate but females were observed to become hermaphrodites at low population densities (Bauer, 1987a). The complex reproductive strategy of freshwater pearl mussels includes high fertility levels resulting in a single female producing several million larvae (glochidia) per year (Young & Williams, 1984). In mid- to late summer the glochidia are discharged into the river. A recent study estimated daily peak releases up to 441 million glochidia per day for a Scottish population (Hastie & Young, 2003b). The proportion of adults producing glochidia is relatively high even in sparse populations (Young & Williams, 1983; Hastie & Young, 2003b; Schmidt & Wenz, 2000; Schmidt & Wenz, 2001), and, therefore, reduced fecundity does not seem to be a limiting factor connected with the lack of juvenile recruitment in most pearl mussel populations.

Viable freshwater pearl mussel populations are highly dependent on viable host fish populations. In the first stage of the life-cycle after their release, the glochidia of *M. margaritifera* must be inhaled by a suitable host fish where they live encysted as obligate gill-parasites for a period of up to 10 months (Bauer, 1994). Glochidia only remain infective for a few days and over short distances downstream the sites from where they are released (Jansen *et al*., 2001). Only sea trout (*Salmo trutta* f. *trutta*), brown trout (*Salmo trutta* f. *fario*) and Atlantic Salmon (*Salmo salar*) are known to host complete metamorphosis in Europe, where they are the only native host species (Young & Williams, 1984). Salmon appear to be the

main hosts in Nova Scotia (Cunjak & McGladdery, 1991) and Russia (Ziuganov *et al*., 1994). In central Europe, brown trout are reported to be the preferred host, or are the only available hosts (Bauer 1987b, c; Wächtler *et al*., 2001). Glochidial rejection is not only limited to non-host fish. Many fish hosts become progressively resistant to glochidial infection (Young & Williams, 1984, Bauer & Vogel, 1987, Ziuganov *et al*., 1994).

During their post-parasitical phase, juvenile pearl mussels bury themselves into the stream sediments for a period of five years, where they depend on a stable substrate with high sediment quality (Buddensiek *et al*., 1993, Geist, 1999a, b). While the mussels gain from their anchorage on their host gills, it is likely that their host fish may benefit from the reduced suspended organic material in river water by filter-feeding by the mussels. Additionally, mussel beds can also provide important microhabitats for juvenile salmonids and the aquatic invertebrates upon which they feed (Hastie & Cosgrove, 2001). Ziuganov & Nezlin (1988) thus consider the mussel / fish relationship to be a variety of symbiosis-protocooperation rather than simple parasitism.

Despite the fact that the status of host fish populations is the first critical step in the life cycle of freshwater pearl mussels, with a high impact on the reproduction of endangered pearl mussels, there is a distinct lack of field data on fish communities from individual pearl mussel rivers, and little is known about the relationship between host stock sizes and the reproductive success of mussels (e.g. Chesney & Oliver, 1998).

This study was conducted in order to compare and assess the status of host fish populations and accessory species in functional (with recent reproduction of *M. margaritifera*) and non-functional (with a lack of juvenile *M. margaritifera*) pearl mussel streams of the Elbe, Danube, Rhine, Weser, Aulne, Kemijoki and Tuuloma drainage systems, to test the hypothesis that the status of host fish populations is linked with the lack of recent pearl mussel reproduction. These results also provide basic data for conservation biologists on the natural abundance and densities of fish communities in stocked and unstocked brown trout streams. Since recent significant changes in wild salmonid stocks may in some areas seriously threaten pearl mussel populations (e.g. Chesney & Oliver, 1998, Hastie & Cosgrove, 2001), such reference data will be of special importance for the ongoing species conservation efforts and for monitoring the effects of natural and anthropogenic impacts which are linked with habitat alteration and management implications.

7.3 Material and Methods

Sampling area

Altogether, 38 surveys using electrofishing were carried out at 36 sites of 20 extant pearl mussel populations from the drainages of Elbe (8 populations), Danube (6 populations), Rhine (1 population), Weser (1 population), Aulne (1 population), Kemijoki (1 population) and Tuuloma (2 populations), including samples from the countries of Germany, the Czech Republic, France and Finland (Table 7.1, Figure 7.1).

Eight of the 20 rivers investigated have regularly or occasionally been stocked with farm hatched *S. trutta* since 1998, mainly as a measure for pearl mussel support. However, no stocking activities were carried out in the investigated streams in the year of the investigation (for at least 6 months before investigation), thus excluding the effects of recent stocking on the evaluation of results.

Only five of the 20 analysed pearl mussel populations still have a significant number of juvenile mussels (>5%) younger than 20 years present, whereas non-functional populations were extremely overaged (youngest mussels usually around 30-50 years). According to Young *et al*. (2001b) an ideal pearl mussel population should have around 20% mussels of less than 20 years old and at least some mussels below 10 years old, a criterion which was met only by the Lutter, the Pikku-Luiro and the Blanice population. Two pearl mussel populations from Kuutusoja and Ruohojärvenoja had significant numbers of juvenile mussels in some parts of the river and are therefore considered to be at least partly functional. It has to be noted, however, that the assessment of functionality and viability of pearl mussel populations is more complex than using the criteria suggested by Young *et al*. (2001b) and that sampling bias during field surveys can occur for young and small mussels (Hastie & Cosgrove, 2002). In order to reduce this error and improve comparability of results, all streams investigated in this study were surveyed using consistent methods.

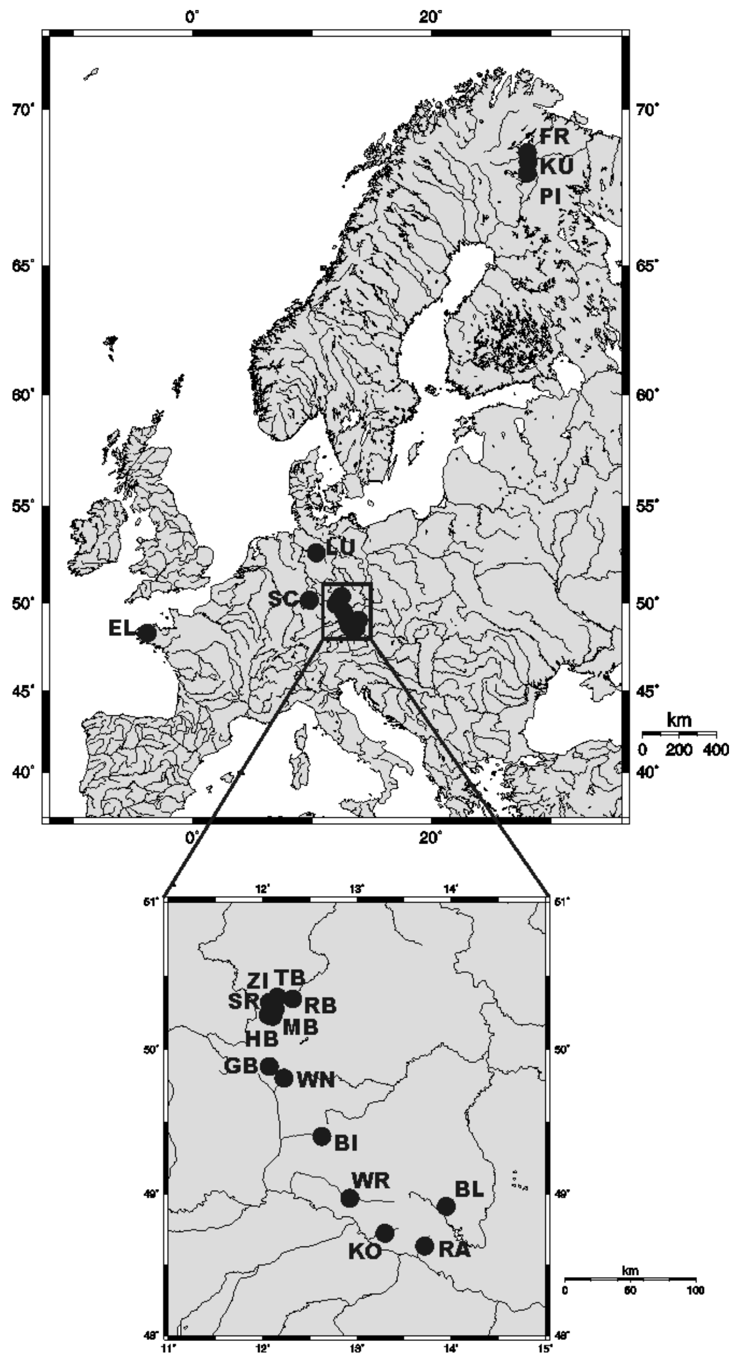


Fig. 7.1 Electrofishing sites (black circles) in European freshwater pearl mussel streams; sample codes according to Table 7.1

Drainage	*Subdrainage*	*Population*	*Code*	*Country*	N_c	*Juvenile Mussels < 20 yr*	*Electric conductivity [µS/cm] ± SD*	*pH ± SD*	*Trout Stocking*	*Number of sites*	*Total number of fishings*	*Year(s) of electro fishing*	*Total stretch length [m]*	*Surface area [ha]*
Elbe	Sächsische Saale	Zinnbach	ZI	D	7,000	-	90 ± 16	6.7 ± 0.3	-	3	4	02/03	455	0.07
	Sächsische Saale	Südliche Regnitz	SR	D	13,000	-	163 ± 37	7.3 ± 0.3	-	3	3	03	300	0.02
	Sächsische Saale	Wolfsbach	WB	D	2,100	-	170 ± 43	7.1 ± 0.4	-	1	2	03/04	250	0.11
	Sächsische Saale	Höllbach	HB	D	34,000	-	89 ± 14	6.8 ± 0.4	-	1	1	02	165	0.04
	Sächsische Saale	Mähringsbach	MB	D	11,000	-	95 ± 14	6.8 ± 0.2	-	1	1	02	100	0.02
	Weiße Elster	Triebelbach	TB	D	<50	-	208 ± 30	6.7 ± 0.3	+	1	1	03	110	0.02
	Weiße Elster	Raunerbach	RB	D	<50	-	189 ± 20	7.1 ± 0.2	+	2	2	03	150	0.05
	Moldau	Blanice	BL	CZ	50,000	++	62 ± 10	6.8 ± 0.2	-	1	1	96	300	0.18
Danube	Naab	Waldnaab	WN	D	3,000	-	181 ± 29	7.4 ± 0.4	++	1	1	03	75	0.07
	Naab	Grenzbach	GB	D	<200	-	112 ± 13	6.9 ± 0.4	++	2	2	04	200	0.03
	Naab	Biberbach	BI	D	500	-	108 ± 8	7.0 ± 0.2	++	1	1	03	100	0.01
	Regen	Wolfertsrieder Ba.	WR	D	2,000	-	41 ± 7	6.9 ± 0.2	++	1	1	03	100	0.02
	Gaißa	Kleine Ohe	KO	D	7,000	-	144 ± 23	7.2 ± 0.2	-	1	1	03	150	0.05
		Ranna	RA	D	600	-	86 ± 6	7.2 ± 0.3	++	1	1	03	95	0.04
Rhine	Fränkische Saale→Main	Schondra	SC	D	100	-	192 ± 36	7.7 ± 0.2	++	3	3	02	270	0.10
Weser	Aller	Lutter	LU	D	4,200	++	199 ± 27	7.0 ± 0.2	-	3	3	03	300	0.15
Aulne		Elez	EL	F	2,000	--	100*	6.7*	-	1	1	04	115	0.08
Kemijoki	Luiro	Pikku-Luiro	PI	FIN	50,000	++	10*	7.0*	-	5	5	04	455	0.13
Tuloma	Suomujoki	Kuutusoja	KU	FIN	1,000	+	22*	7.0*	-	2	2	04	200	0.10
	Lutto	Ruohojärvenoja	FR	FIN	1,000	+	16*	7.0*	-	2	2	04	207	0.13

Tab. 7.1: Characterisation of electrofishing sites; Drainages, Subdrainages, Populations, Codes and Countries; N_c= estimates for actual census populations of pearl mussels; Juvenile mussels: - = no juvenile mussels younger than 20 years present; + = 5-20% of total mussel population younger than 20 years; ++= more than 20% of total mussel population younger than 20 years; Arithmetric means for conductivity and pH measured from the years 2000 until 2004 ± standard deviation SD; brown trout stocking (++= annually since 1998; + = occasionally since 1998; - = no stocking since 1998; number of sites investigated; total number of electrofishings; years of investigations; *= only values from Aug-Sept 2004; D=Germany, CZ=Czech Republik; F=France; FIN=Finland

Freshwater pearl mussel distribution range is limited to clear trout streams which are poor in nutrients and lime, resulting in low conductivity levels, usually less than 200 µS cm^{-1} (Table 7.1). pH values in pearl mussel streams are usually neutral to slightly acidic and reached the highest values in the Schondra (mean pH: 7.7).

All electrofishing was carried out from 2002 to 2004 with the exception of the strictly protected Blanice river in the Czech Republic, for which fish data from the year 1996 were made available by J. Hruška. For species protection reasons, it is not possible to provide detailed GPS-coordinates of the sampling sites, but they can be made available on demand by the corresponding author. As pearl mussels generally occur in a patchy distribution pattern, sites for electrofishing were selected in a representative way for the assessment of the fish community in the area of potential natural infection with glochidia, i.e. downstream of the largest mussel beds. In rivers where more than one large mussel bed was found, several stretches for electrofishing were selected downstream of the mussel beds. The length of the stretches used for electrofishing was adapted to the local conditions, to match the potential area in which natural infection with glochidia was expected (Jansen *et al*. 2001). At sites where mussels occurred in places with slower current, or where the natural infection zone was limited by other means, the total length was reduced to a minimum of 50 m, whereas at sites with a dense mussel population over a longer stretch, or at sites with higher current flow, the total length of a single section was extended up to 165 m.

To assess the fish communities at the time of pearl mussel reproduction, the dates for electrofishing were specifically arranged for each river, according to the time of glochidial release of the pearl mussels, which took place from late June to early October. In all cases, the pearl mussel spawning season did not overlap with the brown trout spawning season, ensuring that the data on trout densities and biomass were not influenced by spawning migrants. Pearl mussel streams are often protected and due to species protection and licensing limitations, repeated electrofishings over several years, in order to test the reliability of data, could only be conducted in selected streams and stretches. These data and additional data from electrofishing in similar streams without pearl mussels (data not presented) showed, however, that species richness, trout biomass and trout density in those small streams did not vary significantly between years, except for one stream that dried out in one year (Wolfsbach), where a decrease of fish biomass by more than 75% was found.

Additionally, it was not possible to kill fish, in order to investigate their gills for natural infection rates with pearl mussel glochidia. Water chemistry data were measured during the investigations and time-series were available from the "Bayerisches Landesamt für Wasserwirtschaft" and R. Altmüller.

Electrofishing and data analyses

Fish populations were sampled by electrofishing. When carried out correctly, electrofishing is reported to have little impact on fish and is harmless to pearl mussels (Hastie & Boon, 2001). The length of stretches for electrofishing was determined according to the potential infection area of trout with pearl mussel glochidia. Stream width was measured along transects every 5 m. Unsuitable habitat within the river bed, such as emergent big stones and extremely shallow zones, with a water depth of < 2 cm, were excluded from the calculation of water surface area. Length and width measurements were either carried out one day before the electrofishing or afterwards, in order to prevent the natural distribution of fish being influenced.

Electrofishing was conducted with different but comparable equipment, produced by Grassl and Efko. Due to the low conductivity levels in pearl mussel streams, the choice of equipment with a high performance of 8-11 kW allowed reliable sampling. Smaller battery dependent equipment was only used in very small brooks with an average width of less than 1.5 m. Prior to sampling, every selected fishing area was enclosed carefully with fine meshed stop nets (6 mm mesh width) at the upstream and downstream ends, to prevent movement of fish into or out of the sampling stretch during the investigation. Electrofishing was carried out by one man wading upstream accompanied by 1-2 people with handnets. In the very small headwater streams, some sites could be fished from the banks. Due to the shallow and clear water and the very limited width of pearl mussel rivers, fishing could be conducted in many separate runs until the sections were completely or almost depleted for trout, which was usually achieved after 2 – 4 successive removal runs. Therefore, all data refer to the cumulative catch and actually represent minimum numbers. The numbers for brown trout are believed to be accurate, as almost no fish were missed due to the high number of separate runs, the high catching effectiveness for trout, and the shallowness and excellent visibility in those clear streams.

Fish from each electrofishing run were kept separate in oxygenated water tanks. Total length (to the nearest mm) and weight (to the nearest g) were measured for each individual. All fish were then released into the same river stretch that they were taken from. The age of fish was determined by analyses of length frequency distribution and was additionally checked by scale reading. Species richness, total biomass, host fish (brown trout) biomass, host fish density, density of 0+ trout, and the biomass percentage of stenoecious fish (*Salmo trutta*, *Lota lota*, *Cottus gobio*) was calculated on a stream basis. For brown trout, length-frequency diagrams were calculated and the number and percentage of yearlings (0+) determined, as an indicator for the intactness of brown trout age structure and reproduction of trout. 0+ trout and 1+ trout lengths could be clearly separated in all of the study streams, whereas size ranges of older fish tended to overlap.

The effect of stocking on host fish biomass and host fish densities, and the differences in host fish biomass and host fish densities between functional and non-functional pearl mussel populations, was tested with two-tailed t-test and its non-parametric counterpart, Mann-Whitney Test. Correlations between fish species richness and host fish biomass and between fish species richness and density of host fish were tested with non-parametric Spearman Rank Correlation analyses. Additionally, regression analyses were carried out to investigate the effect of pH and conductivity on total fish biomass. Classical procedures require distributional assumptions, which are usually met by a large sample size. When the sample size is small the data structure does not always conform to the parametric assumptions; hence the use of non-parametric test in this study. For the same reason newer resampling methods were tested and they appeared to give results that were between parametric and non-parametric results, being closer to latter methods.

To assess the influence of the time of the year in the investigation periods, regression analyses of all electric fishing events were used to test for effects of the month of fish investigation on the density of host fish , the density of host fish fry and the percentage of host fish fry.

7.4 Results

Species richness and fish biomass

A total of 26 fish species, one lamprey and one crayfish species were caught in the 20 pearl mussel streams investigated in this study. The species richness among rivers varied widely, covering a range of two to 16 species per stream (Table 7.2). The majority of pearl mussel streams yielded two to four species only, usually comprising brown trout (*Salmo trutta*), bullhead (*Cottus gobio*) and/or brook lamprey (*Lampetra planeri*). Streams with low numbers of fish species were often found in the Danube drainage and in the headwater streams of the Elbe drainage system. The maximum species diversities occurred in the Südliche Regnitz (12 species), the Waldnaab (16 species) and the Lutter (14 species), and often resulted from a high percentage of ubiquitous species which are atypical for the trout region, such as Cyprinids. In some cases, a high species richness is influenced by escaping fish from fish hatcheries or fish ponds, as species like tench (*Tinca tinca*), pike-perch (*Sander lucioperca*), carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*), are highly unlikely to reproduce successfully in these areas.

With exception of the Waldnaab, brown trout (*Salmo trutta*) were found in all of the investigated pearl mussel streams but showed highly variable density and biomass values between rivers. Bullhead occurred in 70% of the pearl mussel populations, with the exception of uncolonized areas (Suomujoki and Lutto catchments in Northern Finland), three populations from the Danube drainage and one population from the Elbe drainage, and were usually found in all age classes, indicating continuous reproduction. The brook lamprey was an accessory species in 55% of all streams, being abundant in all of the Elbe populations, the Lutter and the Schondra, but found in only one Danubian population. Roach (*Rutilus rutilus*) and perch (*Perca fluviatilis*) were found in 35% and 30% of the streams, respectively, but usually occurred in low densities. In contrast, minnows, where present, occurred in high numbers and were found in 25% of the investigated streams. They were completely absent all of the Danube streams investigated in this study. Grayling (*Thymallus thymallus*) was also caught in 25% of the streams, mostly at sites where pearl mussel distribution extends below the headwater trout regions. The European eel (*Anguilla anguilla*), considered to be a potential predator for juvenile pearl mussels, was only abundant in the Schondra population

(77 kg ha^{-1} and 14% of total fish biomass), but was also found in very low densities in one Elbe stream, the Lutter and two Danube populations, where it is a non-native species.

The fish species in rivers with reproducing pearl mussel populations covered a range of three to 14 species. In all of them, brown trout dominated. Accessory species also varied between functional pearl mussel rivers, with the following species found in more than one river: Minnow, bullhead, roach, pike (*Esox lucius*), burbot (*Lota lota*), grayling and brook lamprey. It is also notable that these functional pearl mussel populations cover the complete range of mean conductivity levels, from 10µS/cm in the Pikku-Luiro to 199 µS/cm in the Lutter.
Total fish biomass ranged between four and 546 kg ha^{-1}, averaging 156 kg ha^{-1}. The lowest values were found for streams in the functional pearl mussel populations in Finland, where streams have the lowest conductivity levels and are extremely oligotrophic. The highest values were obtained for the Biberbach and the Schondra from the Danube and Rhine drainages, respectively. The impact of pH on fish biomass is clear (p-value for slope = 0.012) although there are other factors involved ($r^2 = 0.32$). No influence of conductivity could be confirmed, implying that slightly enriched pearl mussel streams do not necessarily yield higher fish biomass. In fact, the Wolfertsrieder Bach shows the lowest conductivity levels of all rivers, except those in Finland, but is among the rivers with highest total and host fish biomass.

Total fish biomass did not correlate with species diversity, measured by the number of fish species ($p = 0.78$). Maximum sizes of brown trout and total fish biomass showed a positive correlation ($r_s = 0.68$, $p = 0.0019$) and both can be interpreted as indicators of the productivity of the stream sections. In most streams, a high percentage of total biomass was attributed to stenoecious species which are typical for the headwater regions, such as brown trout, bullhead and burbot. These species accounted for less than 50% of fish biomass in only four streams (Südliche Regnitz, SR; Waldnaab, WN; Elez, EL; Kuutusoja, KU).

Non-native species were detected in 20% of the streams and mostly coincided with fish farming ponds in the upstream regions. Non-native fish were found in the Danube headwaters and in the Lutter, and usually occurred in low frequencies only. Competition between native brown trout and non-native rainbow trout can be excluded for all investigated rivers. In the Biberbach, the alien crayfish *Pacifastacus leniusculus* was found in high densities with a minimum biomass of 185 kg ha^{-1}.

		Elbe								Danube						Others							
		ZI	SR	WB	HB	MB	TB	RB	BL	WN	GB	BI	WR	KO	RA	SC	LU	EL	PI	KU	FR	**Total number**	**Total %**
Anguillidae	*Anguilla anguilla*				+					+				+		+	+					**5**	**25**
Balitoridae	*Barbatula barbatula*															+						**1**	**5**
Cottidae	*Cottus gobio*	+	+	+	+	+		+	+			+	+		+	+	+	+	+			**14**	**70**
Cyprinidae	*Abramis bjoerkna*																+					**1**	**5**
	Abramis brama		+														+					**2**	**10**
	Alburnoides bipunctatus									+												**1**	**5**
	Alburnus alburnus									+												**1**	**5**
	Barbus barbus									+												**1**	**5**
	Chondrostoma nasus													+								**1**	**5**
	Cyprinus carpio		+																			**1**	**5**
	Gobio gobio		+							+				+			+					**4**	**20**
	Leuciscus cephalus		+							+				+		+						**4**	**20**
	Leuciscus leuciscus		+							+							+	+				**4**	**20**
	Phoxinus phoxinus		+						+								+			+	+	**5**	**25**
	Rutilus rutilus		+		+					+							+	+	+		+	**7**	**35**
	Tinca tinca									+												**1**	**5**
Esocidae	*Esox lucius*									+				+			+		+			**4**	**20**
Gadidae	*Lota lota*	+	+																+		+	**4**	**20**
Percidae	*Perca fluviatilis*		+		+					+				+			+	+				**6**	**30**
	Sander lucioperca									+												**1**	**5**
Petromyzontidae	*Lampletra planeri*	+	+	+	+	+	+	+	+	+						+	+					**11**	**55**
Salmonidae	*Salmo trutta f. fario*	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+	+	+	+	**19**	**95**
Thymalidae	*Thymallus thymallus*									+						+	+	+		+		**5**	**25**
Non-native species	*Pseudorasbora parva*									+												**1**	**5**
	Oncorhynchus mykiss									+	+			+								**2**	**10**
	Ameiurus nebulosus																+					**1**	**5**
	Pacifastacus leniusculus[1]											+											
																						Mean ± SD [min-max]	
Total number of species (fish and lampreys)		4	12	3	6	3	2	3	4	16	2	2	2	8	2	7	14	6	5	3	4	**5.4 ± 4.1 [2 – 16]**	
Total fish biomass [kg/ha]		87	252	123	55	133	63	155	n.d.	118	60	481	311	151	237	546	68	63	28	4	23	**156 ± 150 [4 – 546]**	
Host fish (*Salmo trutta*) biomass [kg ha^{-1}]		77	51	117	44	121	63	141	n.d.	0	32	478	307	126	234	381	36	25	15	2	16	**119 ± 136 [0 – 478]**	
Percentage of host fish biomass of total		89	20	96	81	91	100	91	n.d.	0	55	100	99	84	99	70	53	40	55	36	70	**70 ± 30 [0 – 100]**	
Percentage of biomass(*S. trutta.C. gobio.L. lota*)		100	23	100	87	100	100	100	n.d.	0	55	100	100	84	100	71	56	43	71	36	80	**74 ± 31 [0 – 100]**	
Minimum size of *S. trutta*		4.5	5.5	2.0	6.0	5.0	4.0	3.0	6.0	n.d.	4.5	6.0	5.2	5.5	5.5	8.0	4.5	11.0	9.5	5.0	5.5	**6.1 ± 2.2 [2.0 – 6.0]**	
Maximum size of *S. trutta*		26.0	29.0	23.0	29.0	35.0	19.0	26.0	25.0	n.d.	23.0	53.0	36.0	28.0	41.0	42.0	37.0	33.0	21.5	17.0	28.0	**30.2 ± 9.1 [19.0 – 53.0]**	
0+ *S. trutta* Individuals ha^{-1}		2496	416	1001	354	1892	4242	613	439	0	149	3387	3821	388	848	105	775	0	0	21	27	**1049 ± 1362 [0 – 4242]**	
1++ *S. trutta* Individuals ha^{-1}		1743	776	4765	859	2054	2259	3020	128	0	484	5323	4450	1659	4347	2716	404	472	455	62	267	**1812 ± 1746 [0 – 5323]**	
S. trutta density [individuals ha^{-1}]		4239	1191	5766	1213	3946	6501	3632	567	0	633	8710	8271	2047	5195	2821	1179	472	455	83	294	**2861 ± 2786 [0 – 8710]**	
Percentage of 0+ individuals of total *S. trutta*		59	35	17	29	48	65	17	77	n.d.	24	39	46	19	16	4	66	0	0	25	9	**31 ± 23 [0 – 77]**	
Number of host fish per 100m river length		67	43	52	29	73	107	111	34	0	9	126	197	63	200	99	58	33	13	4	5	**66.2 ± 58.9 [0 – 200]**	

Tab. 7.2: Characterisation of fish species distribution in 20 European freshwater pearl mussel rivers; [1] crayfish not considered for species number and biomass calculations; river codes according to Table 7.1

Host fish biomass and host fish density

Host fish biomass averaged 119 kg ha^{-1} but varied considerably between rivers (Table 7.2). It showed a strong positive correlation with total fish biomass ($r_s = 0.84$, p = 5.8E-06). This can be explained by the fact that brown trout are the dominating fish species in most of the investigated pearl mussel streams. Only in four streams (Waldnaab, Südliche Regnitz, Elez, Kuutusoja) did brown trout contribute less than 50% of total fish biomass. Host fish biomass showed a highly significant correlation with host fish density ($r_s = 0.86$, p = 1.8E-06). The density of brown trout ranged between 0 and 8710 individuals ha^{-1} for all populations. In functional pearl mussel populations, the density of host fish varied at a lower level between 83 and 1179 individuals ha^{-1}.

Streams where regular trout stocking takes place tended to have higher trout biomass (t-test p = 0.053, Mann-Whitney p = 0.069) and densities of host fish (t-test p = 0.062, Mann-Whitney p = 0.090) than natural populations (Figure 7.2A). In fact, the three populations yielding the highest densities of brown trout (Biberbach, Triebelbach, Ranna) and the five populations yielding the highest brown trout biomass (Biberbach, Schondra, Wolfertsrieder Bach, Ranna, Rauner Bach) are all annually or occasionally stocked. On the other hand, trout stocking seems to have no or limited success in the Waldnaab and the Grenzbach. Despite the fact that large numbers of host fish are annually stocked in those two streams, their biomass and densities are zero (Waldnaab) or at the lower limit of all other rivers (Grenzbach). For the Waldnaab, the results of the electrofishing and the complete lack of host fish in the pearl mussel distribution range were also confirmed by additional electrofishings in other years (Ring, pers. comm.).

Surprisingly, functional pearl mussel populations, where juvenile pearl mussels can still be found, had significantly lower densities of host fish (t-test p = 0.00080, Mann-Whitney p = 0.010) and lower trout biomass (t-test p = 0.0036, Mann-Whitney p = 0.018) than populations with a lack of pearl mussel reproduction (Figure 7.2B). Low biomass and densities of host fish were especially found in Finnish pearl mussel streams, which are extremely poor in nutrients. Among the three functional pearl mussel populations from Finland, the Pikku-Luiro population is largest and additionally has the highest percentage of juvenile mussels younger than 20 years (Table 7.1). Simultaneously, Pikku-Luiro population showed the highest density of brown trout, indicating that host fish may be the limiting factor for pearl mussel reproduction in this area.

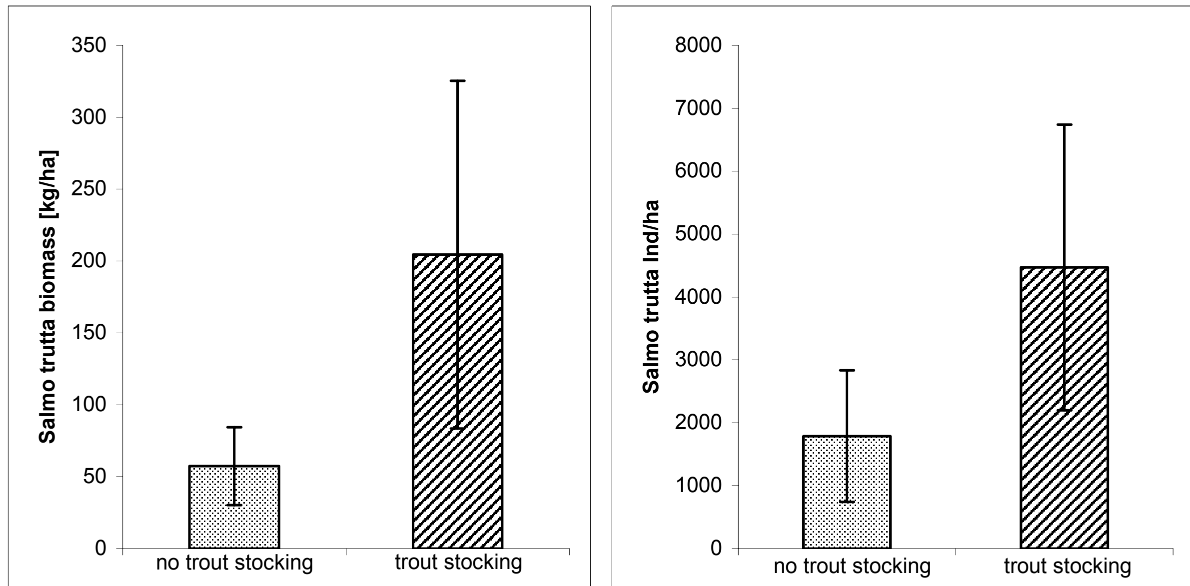
The densities and percentages of young-of-the-year (0+) brown trout, as an indicator for the reproduction of the host fish, varied strongly in both functional and non-functional pearl mussel populations and averaged 252 0+ trout ha^{-1} (35%) in functional and 1314 0+ trout ha^{-1} (30%) in non-functional populations. The highest percentages of 0+ brown trout in pearl mussel streams were found in Blanice (77%) and Lutter (66%), which are both functional populations with high sediment quality. A complete lack of 0+ brown trout in the vicinity of mussel beds was only detected in two populations, in the Elez (non-functional mussel population) and in the Pikku-Luiro (functional mussel population). The Elez population is locked between two dams, and the poor status and reproduction of brown trout is probably caused by strong fluctuations of water-runoff and flow current in this area.

A different situation was observed at the functional Pikku-Luiro population in Lapland, where only 1++ host fish were found during 5 electrofishings at 5 sites, covering almost the complete pearl mussel distribution area in this river. In this case, intensive electrofishing at additional sites within the same river (data not presented) revealed a high number of 0+ fish, which presumably had a different habitat preference and only occurred at remote downstream sites. Therefore, a low percentage of 0+ host fish in the potential glochidial infection area does not automatically imply a lack of reproduction of host fish and a limited reproduction potential for pearl mussels. Taking all sites at all investigated streams into account, no significant lack of specific age classes of brown trout was observed in specific regions, except for the Elez. Together with the pH values (Table 7.1) of these poorly buffered siliceous streams, this indicates that none of the investigated pearl mussel streams has recently suffered from acidification effects, which would usually happen during snowmelt-runoff in spring.

Increased species richness coincides with a reduced host fish biomass ($r_s = -0.39$, $p = 0.097$; Figure 3A). This pattern is even more pronounced for the negative correlation between the total number of fish species and the number of host fish individuals ($r_s = -0.57$, $p = 0.0088$; Figure 3B).

None of the results were significantly affected by the time of year that the investigations took place.

A



B

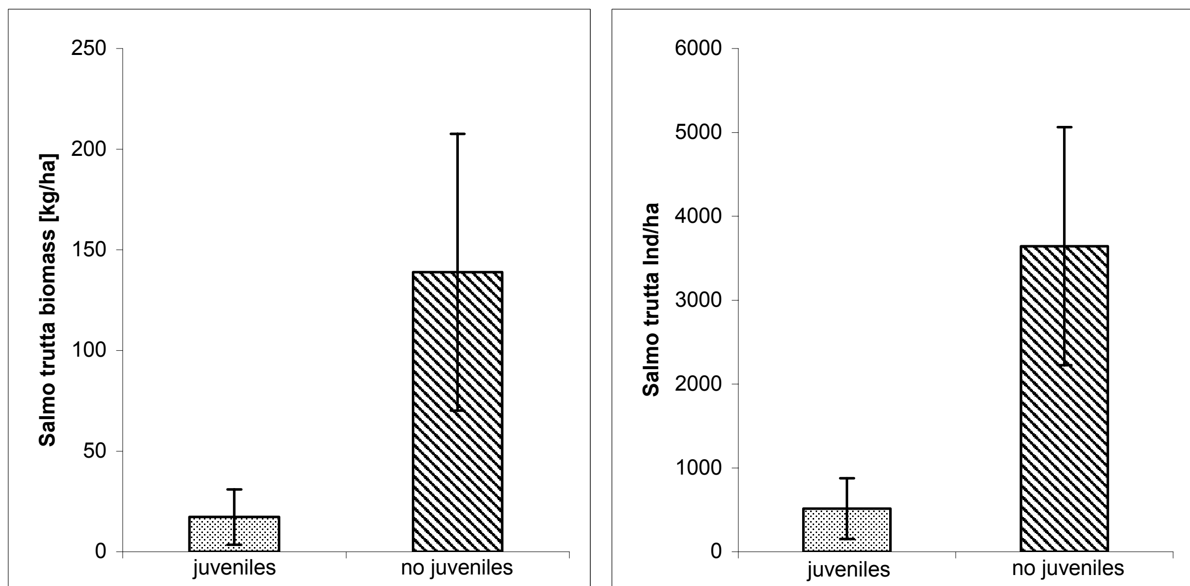


Fig. 7.2: Comparison of *Salmo trutta* biomass and densities between streams with and without trout stocking (A) and between rivers with and without reproduction of freshwater pearl mussels (B); arithmetric means ± 95% confidence intervals

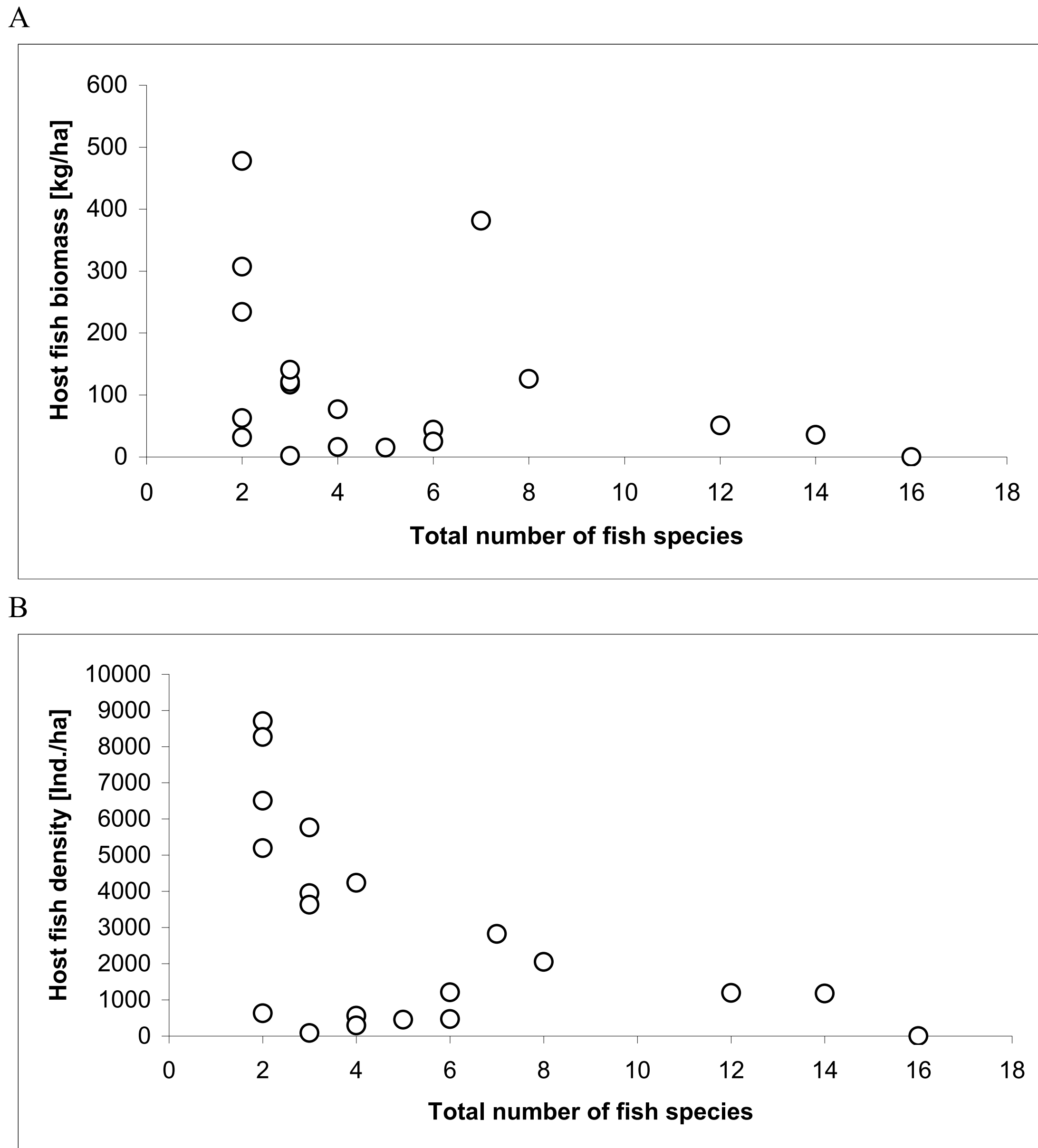


Fig. 7.3: Correlations of species richness with host fish biomass (A) and species richness with host fish density (B) in European freshwater pearl mussel streams

7.5 Discussion

The status of host fish populations

Fish communities of the 20 pearl mussel streams investigated in this study showed considerable variation in species compositions and biomass of host fish and accessory species. In agreement with previous studies of central European populations (Bauer, 1987b, c; Wächtler *et al*., 2001), brown trout were found to be the only available hosts for freshwater pearl mussels in these rivers and neither sea trout nor salmon were detected. The large

northern pearl mussel rivers, i.e. the Tuuloma and the Kemijoki, were important salmon rivers half a century ago. However, this study pertains to present day relationships between pearl mussel recruitment and host densities, therefore the significance of residual dependency on salmon in these rivers is not addressed in this publication.

The density range of brown trout found in this study (mean= 2861 ha^{-1}; range= 0-8710 ha^{-1}) is slightly lower than that of a previous study (Bauer *et al.*, 1991), where an average of 5185 brown trout ha^{-1} and a range of 2000 to 14000 host fish ha^{-1} was observed. Despite the lack of adequately researched host fish densities in pearl mussel streams (Skinner *et al.*, 2003), acceptable levels of brown trout have been suggested to be in the order of 0.1 individuals m^{-2} (Ziuganov *et al.*, 1994) to 0.2 fish m^{-2} (Bauer, 1991), equivalent to 1000 to 2000 fish ha^{-1}. In fact, 80% of the functional pearl mussel populations, but only 33% of the non-functional pearl mussel populations from different geographical regions included in this study, showed lower densities of brown trout than these values. This could either be interpreted as a potential threat to the functional populations due to low host fish densities, or by the explanation that these low host fish densities, coinciding with low productivity and low eutrophication in pristine populations can be sufficient to maintain sustainable pearl mussel reproduction. In fact, functional pearl mussel populations from the Lutter may profit from additional infections of autochthonous fish (Altmüller & Dettmer, 2001) and populations in extremely oligotrophic streams, which were considered to be functional, may depend on the input of migratory fish, which were not detected in any stream during this study. The explanation that low host fish densities may result from sampling in exceptional years is highly unlikely, as electrofishings for both functional and non-functional populations covered different years and fluctuations of fish populations in the small headwater streams, which were sampled in more than one year, were found to be generally low.

The average values of brown trout biomass and brown trout densities in rivers without functional pearl mussel populations were higher than in intact populations. Negative impacts on host fish populations caused by low pH-values during snowmelt-runoff in spring can be ruled out for nearly all of the investigated rivers. Assuming that the data for functional populations are representative, a poor status of host fish can only explain the lack of juvenile reproduction of freshwater pearl mussels in a very limited number of streams (e.g., the Waldnaab, the Grenzbach and the Elez).

However, it is impossible to provide universal minimum values for the required density of host fish in intact pearl mussel populations, as such values will depend on several variables. Firstly, the distribution pattern and the distribution area of mussels in a specific river (normally correlated with the population size) and its current will largely influence the number of host fish exposed to glochidia and thus influence the number of necessary host fish. A long life span and thus long reproductive activity can be expected to reduce the number of host fish required for sustaining a population.

Furthermore, different mortality rates of pearl mussels during other critical stages of their life cycle before and after the parasitical phase, e.g. survival and dispersal of host fish bearing glochidia until drop-off and mussel survival rates during the postparasitical phase, will differ between rivers. In functional, pristine pearl mussel populations in Finland, host fish densities and biomass were found to be extremely low. These low host fish densities can probably be compensated for by the prolonged reproductive life span of pearl mussels in these areas and by low mortality rates among juvenile mussels during their post-parasitical phase, due to a high availability of suitable substrate areas (Geist & Porkka, in prep.). Unsuitable sediment conditions are probably the most critical factor in the life cycle of central European populations (Buddensiek *et al*., 1993; Geist, 1999a, b). In rivers where only few sites have a bottom substrate suitable for the development of juvenile mussels, a higher density of host fish will be required to compensate for the mortality of the individuals that drop off at unsuitable sites.

Additionally, different susceptibilities and immunity reactions of different brown trout strains can be expected. Such differences in the intensity of glochidial infestation have been documented between individuals (Young & Williams, 1984; Bauer & Vogel, 1987), and populations or races (Wächtler *et al*., 1987; Engel & Wächtler, 1989; Hochwald, 1997). This is in contradiction to laboratory experiments, where different strains of 0+ trout (*Salmo trutta* forma *fario* and *Salmo trutta* forma *lacustris*) yielded similar numbers of 236 and 272 viable mussels, respectively, after initial infection with 500 to 1000 glochidia per fish (M. Lange, pers. comm.).

The age and length distribution of fish will also play a major role. On the one hand, immunity reactions after progressive infection with glochidia will decrease the chances of older trout being suitable hosts (Young & Williams, 1984; Bauer & Vogel, 1987; Ziuganov *et al*., 1994).

On the other hand, bigger host fish pump more water through their gills and will therefore receive more glochidia (Bauer & Vogel, 1987). In fact, older trout can be successful hosts if they have had limited previous contact with glochidia. For rivers in Northern Germany and Scotland, big host fish (even >3+) were exceptionally found to carry several thousand glochidia (e.g., Altmüller & Dettmer, 2001; Hastie & Young, 2003b). In rivers with a high infection rate, the effects of increasing immunity and higher glochidia carrying capacity will probably compensate for each other, a situation that is supported by a field study in the Wolfsbach by Schmidt *et al*. (2000), where 1-2031 glochidia per trout were found, independent of the size of the fish. These results are also supported by a study from Young *et al*. (1987), who found an average of 1333 encysted glochidia 190 days post-infection and were able to successfully reinfect their host fish in a second year. Therefore, the density of host fish individuals related to the available water surface may be the most suitable proxy for the assessment of the chances of glochidia successfully encysting on the host gills.

In populations where only older or mainly older host fish are found in the areas downstream of mussel banks, and where spawning and rearing places for yearling fish are situated in other regions, the availability of bigger fish may compensate for the low numbers of individuals available (e.g. Northern Finland). Studies on infection rates of trout in different streams shortly after infection and shortly before the drop-off of glochidia would allow a more rigid evaluation of the effects of natural and aquired immunity and the suitability of different age classes of host fish in natural populations. Such studies, however, require the killing of host fish and cannot currently be carried out in most of the endangered pearl mussel populations.

The results of this study also show that increased fish species richness in pearl mussel streams coincides with reduced biomass and density of host fish. An increase in non-host fish species will increase the number of glochidia that contact non-host fish, where they cannot develop and this in turn will reduce the contacts between glochidia and suitable host-fishes. Most freshwater pearl mussel streams are extremely oligotrophic with a small number of specialised fish like salmonids, which maximises the chances for glochidia to attach to the gills of a suitable host. It seems that eutrophication and other anthropogenous activities linked with habitat degradation can actually reduce competitiveness of brown trout and result in an increase of the abundance, densities and biomass of less specialised species. This observation can be disguised by the fact the occurrence of a larger spectrum of species is expected to be natural in some pearl mussel populations, particularly those with distributions in lower

reaches of rivers. In most situations, host availability is likely to be a far more important factor than overall glochidial production or the competition for glochidia between host fish and non-host fish, considering the enormous numbers of released glochidia and the comparatively limited number of host fish.

An assessment of electrofishing data must consider that fish abundance and distribution can vary considerably between different times of the year. For instance, fry density declines, e.g. due to mortality, predation, and dispersal can occur (e.g. Egglishaw & Stackley, 1977), potentially causing erroneous interpretations when comparing streams that have been electrofished at different times of the year. Electrofishings in this study were specifically carried out at the time of glochidial release and in the potential infestation areas of host fish in order to minimise a sampling bias and allow comparability of the status of relevant host fish between rivers. The fact that no bias in the densities of host fish, host fish fry and the percentage of host fish fry was found in surveys carried out in different months indicates that large differences between the status of host fish populations prevail and that these differences must be attributed to other factors, such as habitat. However, the survival rates and dispersal of infested host fish and fry, e.g. during winter, and the time of the parasitic phase on the host fish may differ between rivers and can have effects on the required density of host fish to sustain pearl mussel population sizes and thus prevents the deduction of a universal benchmark value for host fish densities.

Implications for management and conservation

The decrease of host fish densities and host fish biomass with increasing species richness shows that habitat alteration and the effects of eutrophication tend to decrease competitiveness of specialised salmonids, whereas less demanding species become more abundant. Conservation measures, such as trout stocking, require evaluation on a stream by stream basis. In cases where continuous stocking has not resulted in stable host fish populations, habitat deficiencies are likely to play a major role. In the Waldnaab, high water temperatures during summer, probably influenced by man-made upstream dams, carp ponds and the effects of an artificial lake, can most likely explain the lack of brown trout at pearl mussel sites during summer, despite the fact that large numbers are stocked during springtime. The timing of stocking can be an important factor with regard to availability of hosts. In the Grenzbach, the low number of host fish is probably limited by habitat structure deficiencies, as stream straightening construction measures were carried out at pearl mussel

sites in this river. An improvement of habitat conditions by a more careful water flow management in the first case, and by habitat structure improvements in the latter case, will be necessary before continuing release of glochidia-infected host fish. Several habitat restorations, including artifical instream cover (e.g., Eklöv & Greenberg, 1998) can help increase the density of 0+ trout. The status of brown trout and its natural reproduction in the Elez river in France could probably be improved by a careful management of water runoff, especially between the spawning season and the hatching of fry.

The success of stocking with glochidia-infected brown trout will also be limited in populations which already have a high density of brown trout, near the habitat carrying capacity. It is highly likely that additionally released glochidia-infected brown trout from fish hatcheries will be under severe competition in streams, such as Biberbach, Wolfertsrieder Bach, Schondra, Ranna and Rauner Bach. In streams with a high natural reproduction rate of brown trout there is no need to carry out additional stocking. Instead, it can be recommended to increase the number of glochidia carrying host fish, by infecting wild brown trout captured from the specific streams. These measures require additional efforts for quickly carrying out electrofishings during the time of pearl mussel glochidia release, but may be especially advantageous in streams with small and concentrated or patchy pearl mussel populations.

In streams with a comparatively high density of potential host fish, normal infection rates of host fish but a lack of reproduction of pearl mussels, other factors, especially the suitability of the substrate which strongly influences the mortality rates during the post-parasitical phase after the drop-off from the host fish, should be given priority for conservation and management of the populations.

In some endangered populations, the rivers have been completely searched for mussels and these have subsequently been gathered in beds to allow a more thorough monitoring of those populations. Such measures may on the one hand decrease the rate of hermaphrodites and inbreeding effects, but can on the other hand reduce natural infection rates of host fish, as the total potential infection area and thus the number of infected fish will decrease when mussels are gathered in one patch only.

8 General Discussion

The development of sound conservation strategies for endangered freshwater pearl mussels and other aquatic organisms is complex, and, therefore, several spatial and temporal issues are important. Research and conservation address problems at various levels: problems at the individual and population level, problems at the species level in the entire range, problems of community and ecosystem diversity, as well as problems connected with the overall goal of sustaining global biodiversity. In order to be successful, conservation efforts must be orientated towards preserving the processes of life (Bowen, 1999). The freshwater pearl mussel is a species which offers great potential to meet these challenges and to discuss sustainable conservation strategies in the context of Conservation Genetics and Ecology. Despite the fact that urgent conservation recommendations are needed in order to maintain the last remaining European pearl mussel populations, conservation strategies must be based upon scientific facts.

8.1 Aspects of conservation on the individual and population level

As a first step on the individual and population level, a thorough understanding of the autecology and habitat requirements of pearl mussels is needed in order to be able to evaluate the current habitat quality, including the assessment of anthropogenic impacts. Different habitat requirements must be met during all phases of the species´ complex life cycle, and potential adaptive differences between populations and genetic variability in individuals and populations must also be considered to address these questions thoroughly.

Almost all European pearl mussel populations, even those in nutrient enriched streams or in sparse populations, seem to still have a high proportion of adults producing glochidia on a normal level (e.g. Young & Williams, 1983; Hastie & Young, 2003b; Schmidt & Wenz, 2000; Schmidt & Wenz, 2001). Thus, problems with this initial phase in the life cycle do not seem to be the primary reason for the serious population declines. Given the high reproductive potential of pearl mussels and the fact that no reduction in fecundity of old mussels has been observed, even small and overaged populations that have lacked reproduction for many years can potentially recover after habitat restoration or through supportive breeding measures. The observed metapopulation structure (chapter 4) and investigations into the demographic structure of viable Scandinavian pearl mussel populations

(Figure 2.2; Geist & Porkka, in prep.) suggest that a temporal lack of juvenile recruitment over some years can be tolerated or even normal in long-lived and healthy populations.

Freshwater pearl mussels are excellent indicators for the interaction of different environmental habitat compartments due to their complex life cycle. Their conservation cannot be viewed separately from that of their host fish, and thus a synecological perspective on the interactions between species in the ecosystem is required. There is a distinct lack of field data on fish communities and in adequately researched host fish densities in pearl mussel streams (Skinner *et al*., 2003). Furthermore, the suspicion that effects of acidification in the oligotrophic, poorly buffered pearl mussel streams may have caused extinctions of host fish populations, and a poor knowledge about the relationship of host stock sizes and the reproductive success of mussels (e.g. Chesney & Oliver, 1998) demanded that sound and quantitative investigations be carried out in this field. Indeed the results of this study showed that a complete lack of host fish or severely disturbed host fish populations can occur in specific pearl mussel streams, and these alone are a sufficient explanation for the lack of juvenile recruitment in these populations (chapter 7). However, this study also revealed that the size and composition of host fish populations appears to be limiting for pearl mussel reproduction only in a small number of streams in certain geographical regions. Even comparatively small host fish populations seem to be sufficient to support large pearl mussel populations if habitat conditions during other phases of the life cycle (e.g. substrate quality and stability, and the survival rate during the post-parasitical phase) are optimal. This example clearly demonstrates the need for interdisciplinary research, as one phenomenon – the decline of pearl mussel populations – can be attributed to different and multiple reasons in different geographical regions.

Several studies suggest that the survival rates of pearl mussels during the postparasitical phase are probably extremely crucial and the key issue linked with the lack of juvenile recruitment in most populations (e.g. Buddensiek *et al*.1993; Geist, 1999a, b). The comparatively high host fish densities and intact age structures of host fish populations found for most pearl mussel streams in this study, and the observed poor sediment quality and low rates of exchange between the free water body and the interstitial water in many European pearl mussel streams (Geist & Auerswald, in prep.) support this view. During their long post-parasitical phase in which pearl mussel live buried into the stream substrate for usually five years, pearl mussels depend on a permanently well-oxygenated and stable substrate. These

criteria are rarely fulfilled in central European populations and deserve special attention. In fact, studies into sediment microhabitats of pearl mussel populations at sites with high rates of juvenile recruitment all showed low percentages of fine sediments, high redox potentials and no or only small differences in the chemistry of water taken from different depths of the interstitial zone and from the free water (Geist & Auerswald, in prep.). Substrate factors probably also closely correlate with the productivity and food availability for juvenile pearl mussels, a field which is still poorly investigated and understood.

Conservation and management strategies on a population level when there are certain habitat deficiencies can be overcome by artificial culturing and breeding techniques. For instance, inadequate host fish populations can be bridged by artificial infection of autochthonous host fish, the infection of host fish in hatcheries and the release of infected fish shortly before drop-off of glochidia, or by directly releasing juvenile mussles from artificially infected and farm-reared host fish. Similarly, the culturing of juvenile mussels in cages or artificial bypass-channels with high sediment quality can reduce mortality rates during the post-parasitical phase if sediment quality or stability is not sufficient in the main stream. The feasibility of culturing *M. margaritifera* as a conservation tool has been studied by Buddensiek (1995), Hastie & Young (2003a), and promising results in this field are reported from the Czech Republic (J. Hruška, pers. comm.) and Germany (M. Lange, pers. comm.). However, such conservation strategies are (semi-) artificial and can only be carried out for a small selection of populations. They should be seen as an important but temporary emergency measure to rescue and maintain genetically unique populations and their variability until the natural habitat can be restored.

As different levels of individual or population genetic variability (e.g. heterozygosity, allelic richness) are often correlated with fitness parameters and the ability to adapt to changes in the environment (e.g. Reed & Frankham, 2003), an evaluation of these genetic parameters on an individual and population level can help to develop sustainable conservation, breeding and culturing strategies for the species, and to avoid genetic bottlenecks and founder effects (see chapter 4). The installation of breeding programmes on a genetic basis should therefore consider measures to maintain the genetic identity of evolutionary significant units (ESUs) and conservation units (CUs) on the one hand, and reduce the effects of genetic stochasticity on small populations on the other hand. Generally, careful evaluation of genetic relationships

and habitat suitability are necessary before carrying out stocking activities with freshwater mussels (Geist & Schmidt, 2004).

In many cases, a careful balancing of arguments is mandatory for deducing conservation strategies on a population level from different scientific results, each of them addressing one specific topic. This can be demonstrated well for the practical management issue of whether it is a useful conservation measure to collect mussels from small populations and to put them together into aggregations. Despite the fact that the example is simplified, as further natural and human dimensions, e.g. handling and observation of the mussels, danger of extinction during natural catastrophes or habitat disturbance all need to be considered, different recommendations would be given when considering either ecological or genetic aspects alone instead of including both for the development of sustainable conservation strategies. As is obvious from chapter 7, a dispersed population structure of pearl mussels will largely increase the number of potential host fish infections and thus has positive effects on the number and dispersal of juvenile pearl mussels. On the other hand, from the genetic point of view, the opposite strategy of putting mussels from small populations together in one group may be suggested in order to avoid selfing, the effects of inbreeding and genetic stochasticity on small populations. In many cases, an improved understanding of ecology and ecological habitat changes is essential for managing the genetic diversity of threatened and endangered species properly. Genetic studies can in turn be beneficial for ecological studies. This approach, landscape genetics, promises to facilitate our understanding of how geographical and environmental features structure genetic variation at both the population and individual levels, and has implications for ecology, evolution and conservation biology (Manel *et al.*, 2003).

Monitoring, dating and assessment of past changes in the environment can be a promising approach for detecting, identifying and subsequently investigating the influence of environmental factors that can explain the species´ dramatic declines in specific populations. As demonstrated in this study, long-lived adult pearl mussels themselves with their tree-like annual shell growth increments can be used as an environmental or physiological long-term archive (chapter 6). Patterns of stable carbon $\delta^{13}C$ signatures in annual shell carbonate growth increments were found to be a marker for metabolic activity, as mussels exposed to identical environmental conditions revealed different individual signature patterns extending over several years. Linking these patterns with biological processes of mussel physiology and

growth can reveal insights into the individual performance and overall fitness of mussels. The methodology of mussel shell analyses established in this study may also be useful for other mollusc species and for annual analyses of the temporal dynamics of environmental variables, such as acidification, eutrophication or pollution effects which are similarly recorded and preserved in mussel shell long-term archives (e.g. Carell *et al*., 1987; Lindh *et al*., 1988; Mutvei & Westermark, 2001). As demonstrated in this study, a combination of stable carbon isotope analyses with stable nitrogen isotope analyses of mussel tissues and potential food sources improve our understanding of physiology and food sources for pearl mussels.

8.2 Aspects of conservation on the species level

In addition to regional attempts to protect and support individual pearl mussel populations, it is essential to consider the species´ biodiversity on a more global scale. Conservation resources are limited. Thus, they require priority setting for populations within species and for biogeographic areas within regions, the incorporation of knowledge of evolutionary processes and the distribution of genetic diversity into conservation planning (Moritz, 2002). Characterisation of genetic variability plays a key role in defining strategies for species conservation which, by definition, seeks to protect a threatened gene pool.

As a first step on the species level, detailed survey work to map current populations and to assess their demography and current imperilment status is required. Recent suggestions for monitoring the freshwater pearl mussel are available from Young *et al*. (2003). Among these populations, priority populations for conservation can be selected by a combination of genetic and ecological methods. From the genetic perspective, conservation units (CUs) should be identified (see chapter 4). The conservation goals attributed to the concept of CUs for freshwater pearl mussel populations involve maintaining genetic diversity in the species, combining concepts of minimum viable populations (Soulé, 1987; Nunney & Campbell, 1993), evolutionary significant units, ESUs (Moritz, 1994; Crandall *et al*., 2000), and management units, MUs (Moritz, 1994). Ideally, genetic diversity should be separated into two dimensions, one concerned with neutral divergence and the other with adaptive variation. Most recent conservation genetics research has focused on the use of neutral genetic markers (Hedrick, 2004), which have been developed and applied in this study (chapters 3 and 4). Additionally, coding mitochondrial markers are available for pearl mussels (Geist, 2002). The application of genetic markers for analysing population diversity and differentiation appears

to be especially important among bivalve molluscs, as morphological features can largely depend on environmental variables (e.g. Johnson, 1970; Watters, 1994).

Ideally, no important populations should be missed during investigation in order to be able to assess the contribution of each population to the species´ total diversity and differentiation. As most extant pearl mussel populations are small, critically endangered and strictly protected, negative impacts on the mussels must be excluded by using non-destructive DNA sampling techniques. In this study this challenge was addressed in a combined approach, using tissue from dead individuals found during survey work and by applying a minimal-invasive sampling technique for haemolymph from living individuals (chapter 4).

In addition to the knowledge about the current genetic structure of extant populations, a better understanding of historical processes connected with the species´ phylogeny, phylogeography, colonisation and extinction patterns can be helpful for future conservation strategies. Thus, it is occasionally useful to additionally include samples from extinct populations into genetic studies. The analysis of shell DNA was demonstrated to be possible in this study, but is more complicated than haemolymph or tissue DNA-analyses, and certain precautions are necessary due to the low quantity and quality of shell DNA (chapter 5).

For selection of priority populations for conservation, the ecological aspects of habitat evaluation, eventually including an assessment of the chances for habitat restoration, should be equally included. This process is comparatively easy if conservation units comprise several populations with similar genetic composition. Under such circumstances, it appears to be reasonable to select priority populations with the most intact habitats by indirect means of pearl mussel population size, age structure, or direct means, e.g. sediment quality, host fish densities or landuse in the catchment area. Habitat dynamics, anthropogenic impacts and economic aspects should also be considered. Conservation strategies become more difficult when genetically unique populations with significant contribution to the species´ total diversity coincide with heavily disturbed habitats, a negative evaluation of ecological habitat parameters, e.g. in river catchment areas with intensive landuse. Generally, it is often discussed whether it is more reasonable to focus conservation approaches on single large or on several small populations, the so-called SLOSS-controvery (Simberloff & Aberle, 1982). The results of this study on pearl mussels suggest a more complex discussion of this topic, as

considerable contribution to the species´ genetic diversity and differentiation seems to be attributed to both small and large populations.

In the next step after the selection of priority populations, strategies to maintain the genetic diversity of the priority populations are required in order to retain the species´ evolutionary potential. The most critical task from the conservation genetics point of view is the balancing between avoidance of inbreeding effects on the one hand and outbreeding effects on the other hand, a topic which is even more difficult for a species like the pearl mussel with facultative hermaphrodism. Maintaining genetic variability of pearl mussels in order to avoid the effects of genetic stochasticity on small populations is important and can include the reestablishement of gene flow between closely related populations (see chapter 4), the so-called migration rescue (Lenormand, 2002). On the other hand, gene swamping between evolutionary significant units adapted to specific habitats can have deleterious genetic effects, the so-called migration meltdown (Ronce & Krickpatrick, 2001). Thus, a conservation of genetic diversity of pearl mussels in different regions of their distribution is advisable.

As conservation actions to protect mussels must often be pursued without waiting for research to provide final answers, adaptive management is suggested to be a useful tool (Strayer *et al.*, 2004). However, it also has to be considered that among long-lived and slow-growing species like freshwater pearl mussels, the time lags between a stressor (e.g. habitat loss or restoration) and the appearance of its effect (e.g. population collapse or rediscovery of juvenile recruitment) are long and can disguise the current status of populations and the effects causing the declines or recoveries. Thus, conservation actions without immediate positive effects on pearl mussels must be judged carefully and the interactions with other species and the complete ecosystem should be given priority.

As demonstrated in this study on Conservation Genetics and Ecology of European freshwater pearl mussels, an interdisciplinary approach integrating aspects of conservation genetics and ecology in large geographical ranges is needed in order to deduce sound conservation strategies for pearl mussels, which – on a next level – also have to include human dimensions to become sound management strategies.

8.3 Aspects of conservation of global biodiversity

The monitoring and conservation management of biodiversity above species level is even more complex but also more important than that on the level of a single species, such as the freshwater pearl mussel. In particular the points of how to define priority habitats and species associations are not free from personal opinions. Despite the fact that invertebrate species represent about 99% of animal diversity (Ponder & Lunney, 1999), and the fact that molluscs belong to the second most diverse animal phylum in terms of numbers of described species (Lydeard *et al*., 2004), invertebrate and mollusc diversity is strongly underrepresented in conservation research (Bouchet *et al*., 1999; Clark & May, 2002; Lydeard *et al*., 2004). Recently, 25 locations were identified as global hotspots for conservation prioritisation, and it was suggested that the limited conservation resources available should be put into these areas first (Myers, 2003). These hotspots were identified using areas with high levels of species endemism in plants, mammals, birds, reptiles and amphibians, but invertebrate diversity is not even specifically mentioned. Such approaches of grossly disproportionate distribution of taxonomic effort towards vertebrates and higher plants (Gaston & May, 1992) remain questionable, since an Australian study showed that invertebrates can be strong predictors for conservation priorities for vertebrates, but not vice versa (Moritz et. al., 2001).

It is often suggested to focus conservation efforts on indicator, flagship, umbrella or keystone species. Some species fulfill one or two of these conditions; some even none. The freshwater pearl mussel can be seen as an exception, as this species at least partly matches criteria involved in all of these concepts.

M. margaritifera can be seen as an indicator species, as it is a stenoecious species which is adapted to cool, oxygen-saturated running waters which are low in lime and nutrients. Pearl mussels are easy to identify and occur in a wide geographic range. They have a complex life cycle, they are long-lived and they are particularly sensitive to eutrophication and other changes in water quality. Although pearl mussels do not appear to be indicators for fish species richness in headwater regions (see chapter 7), they are good indicators for the co-occurrence of specialised species, ecosystem health and functioning (e.g. nutrient cycles), and structural diversity, being important factors e.g. for their fish hosts and for a series of accessory species, such as lampreys and the larvae of ephemeropterans, trichopterans and plecopterans.

A conservation strategy for umbrella species is orientated towards providing sufficiently large areas for species with a wide home range, also bringing other species under that protection. The factors which control mussel populations can arise at various distances from the mussels (Strayer *et al*., 2004). While local conditions are undoubtedly important for mussels, more distant factors, such as geology and land use in the catchment area, may have strong effects as well. Additionally, it seems that functional pearl mussel populations match a metapopulation model in many areas, implying positive effects of gene flow between subpopulations within evolutionary significant units of interconnected river systems (see chapter 4). This largely depends on the existence of intact river systems without artificial barriers (e.g. man-made dams or sewage inputs) that hamper or prevent the migration of host fish vectors. Thus, pearl mussel conservation is a wide-ranging conservation approach, matching the ideas underlying the concept of an umbrella species, although extant pearl mussel populations are most often only limited to small patches in the headwaters of streams.

The freshwater pearl mussel has become a popular symbol and leading element of entire conservation campaigns, attributed to the concept of flagship species. Despite the fact that the species is not as charismatic as large vertebrates, the pearl mussel is identified with pristine and healthy stream ecosystems and has been used as a poster-animal, e.g. on stamps in Germany and the Czech Republic. The cultural and historical importance of the species producing valuable pearls may contribute to the symbolic character.

It has to be considered that single species management of flagships, umbrellas, endangered species and others can lead to the odd circumstance that their management conflicts with the management of another species (Committee on Scientific Issues in the Endangered Species Act, CSIESA, 1995), and that single species management of an indicator species by means of only supporting this species with semi-artificial measures is a self-contradiction (Simberloff, 1998). Conservation strategies addressed towards a rescue of sustainable pearl mussel populations will require habitat restoration and will also benefit its host fish and a series of similarly vulnerable but less popular species, which matches the idea of functional keystone species.

The concept of the keystone species suggests that certain species have impact on many others, often far beyond what might have been expected from consideration of their biomass or abundance. The original definition of ´keystone´ has been expanded (Bond, 1993;

Menge *et al*., 1994), and species that are not near the top of foodwebs have also been seen as keystones. Thus, the freshwater pearl mussel may ideally match the ideas behind the concept of keystone species. Changes of the physical structure of stream sediments by dense mussel populations, their effects on water clearance, light penetration, abundance of macrophytic plants, and the resultant increase in aquatic organisms dependent on these structures for attachment, food or cover, are examples which illustrate that freshwater bivalves in general and freshwater pearl mussels in particular can be viewed as keystone fauna of aquatic ecosystems, their presence greatly enhancing biodiversity.

Given the suitability of pearl mussels as flagship species on the one hand, and their important ecological functions as indicator, keystone and umbrella species on the other hand, they can be seen as an ideal target species for practical conservation efforts in stream ecosystems.
Generally, conservation priorities should move away from simply species- and habitat-orientated goals towards the idea of conserving the evolutionary process on which all biodiversity depends.

8.4 Recommendations for future research

Future pearl mussel research on the individual and population level should particularly focus on the habitat requirements of juveniles during their post parasitical-phase, including studies on sediment quality, dynamics and their influences on the food webs. The use of stable isotope analyses demonstrated in this study suggests a range of extended applications to assess the food quality and quantity requirements for juvenile and adult pearl mussels. Our understanding of adaptation and of the interactions between genotypes and environments can be improved by combining molecular genetic techniques with physiological and metabolic analyses (e.g. stable isotope methods) to investigate the functional link between genotypes and fitness parameters under different environmental conditions. These aspects will also be important for establishing sound breeding and culturing programmes for specific populations. Another main task will be to assess the long-term dynamics and viability of long-lived pearl mussel populations in correlation with their evolutionary potential, and to use ecological and genetic methods to understand the importance and interactions of multiple controlling factors with distribution and population structure of pearl mussels and their fish hosts. In particular, the influence of stream hydrological processes on microhabitat, particularly hydrodynamic effects on juvenile recruitment, is poorly understood (Skinner *et al*., 2003). Modelling the

pathways of water runoff, nutrients and stressors in the catchments are an important component for carrying out effective stream habitat restoration measures.

On the species level, further survey work on the distribution and status of pearl mussel populations is needed. This especially includes making these data available for other researchers in the field. Currently, genetic analyses of samples from many geographical regions are being carried out (Geist *et al*., in prep.). However, it is highly recommended that more populations representative of all different geographical regions are included into genetic investigations to study neutral divergence and adaptive variation of freshwater pearl mussels. Such studies in a more global context will help to identify further priority populations for conservation and retain the maximum evolutionary potential. Genetic studies into *Margaritifera margaritifera* may additionally deliver important contributions to our knowledge about the historical, phylogenetic and phylogeographical processes of post-glacial colonisation patterns.

Above species level, one of the main tasks will be to gain a better understanding of the network of links between pearl mussels with their ecosystem and their importance for global biodiversity. This includes further studies into co-occurrence patterns, the correlation of population fluctuations of pearl mussel and accessory species. C and N stable isotope analyses suggest investigations into the complex interactions of accessory species, food webs and the trophic level organisation in functional and disturbed pearl mussel habitats. Due to their comparatively sessile mode of life and longevity, pearl mussels and their distribution patterns can allow long-term interpretations on habitat factors and stream dynamics, as well. Another interesting research approach will be to resolve the link of patterns in the genetic structure between pearl mussels, their fish host vectors and other accessory species, and to assess these data in correspondence with differing life histories, demographic and stochastic effects. Studies into the genetic structure and biodiversity patterns of other freshwater bivalves with different modes of reproduction and in different habitat types can contribute to the understanding on the impacts of inbreeding depression under different reproductive strategies, and they can broaden the view of the genetic and ecological processes upon which mollusc biodiversity depends.

9 Summary

Despite the fact that mollusc species play important roles in many aquatic ecosystems, often little is known about their ecology, biodiversity and population genetics. Freshwater pearl mussels (*Margaritifera margaritifera* L.) are among the most critically endangered freshwater invertebrates, facing serious population declines and local extinctions.

The goal of this study is to contribute knowledge for designing conservation strategies for the species by combining conservation genetics and ecological investigations.

Altogether 14 polymorphic microsatellite markers were developed for *M. margaritifera,* representing the first published microsatellite markers for an European freshwater bivalve mollusc (order Unionoida). The markers revealed wide ranges of allelic richness and heterozygosity levels and proved to be suitable for monitoring of neutral genetic divergence and diversity in order to describe the current genetic structure of pearl mussel populations.

The genetic diversity and differentiation of the last and most important central European pearl mussel populations from the drainages of Elbe, Danube, Rhine, Maas and Weser were assessed in order to determine conservation units (CUs), to select priority populations for conservation, and to deduce conservation strategies on a genetic basis for free-living populations and for supportive breeding measures. A high degree of fragmented population structure and different levels of genetic diversity within populations were detected. This observation can most likely be explained by historic, demographic and anthropogenic effects.

The methodology of non-destructive sampling with no impact on living populations was established for pearl mussel DNA-analyses (dead individuals and haemolymph sampling). In addition, the successful use of shell-DNA was demonstrated. The potential of using mollusc shells for DNA-based analyses and the required precautions and limitations to avoid erroneous results were discussed.

This study also explored the potential of separating annual growth increments of pearl mussel shell carbonate layers, and their suitability as long-term archives for up to 100 years. Stable isotope investigations of inner nacreous and outer prismatic shell carbonate increments demonstrate that pearl mussel $\delta^{13}C$ shell carbonate signatures record individual metabolic signals extending over several years and that a high percentage of respiratory CO_2 (community and mussel respiration) contributed to shell aragonite formation. In combination with $\delta^{15}N$ signatures of mussel tissues and potential food sources, these analyses allowed an assessment of the trophic level and of the origin of the mussel diet.

The study on the status of host fish populations and the fish species richness in European pearl mussel populations characterised typical fish communities in pearl mussel streams and reveals that a lack of host fish only seems to be limiting for pearl mussel reproduction in specific areas. Intact and functional pearl mussel populations were found to occur under extremely oligotrophic conditions with lower host fish densities and biomasses than disturbed central European populations without juvenile recruitment. The effects of stocking measures with glochidia infected host fish as a conservation strategy were discussed.

Due to the pearl mussels´ wide geographical distribution, their complex life cycle and extraordinary reproductive strategy, the global phenomenon of serious decline can have different and multiple reasons in different regions. Conservation strategies in general and those for freshwater pearl mussels in particular can greatly benefit from a holistic and combined approach of integrating conservation genetics and ecological studies to retain a maximum of the species´ biodiversity and evolutionary potential on the one hand, and try to identify their habitat requirements and restore their habitat to meet the specific requirements during all stages of the life cycle on the other hand. Thus, conservation strategies for pearl mussels can be key examples for the development of conservation strategies for other aquatic organisms and the ecosystem functioning upon which they depend. Generally, conservation efforts should move away from single species and pattern protection towards process and persistence conservation for ecosystems.

10 Zusammenfassung

Trotz der wichtigen Rolle, die Mollusken in vielen aquatischen Ökosystemen spielen, ist meist nur wenig über ihre Ökologie, Biodiversität und Populationsgenetik bekannt. Flussperlmuscheln (*Margaritifera margaritifera* L.) zählen zu den am stärksten gefährdeten Süßwasserinvertebraten. Die Art weist starke Bestandseinbrüche auf und stirbt in bestimmten Regionen aus.

Übergeordnetes Ziel dieser Arbeit ist es, mit einem integrierenden Ansatz von molekulargenetischen und ökologischen Untersuchungen zur Entwicklung von Artenschutzstrategien und damit zum Erhalt dieser Art beizutragen.

Insgesamt wurden 14 polymorphe Mikrosatelliten-Marker für *M. margaritifera* entwickelt, die die ersten veröffentlichten Mikrosatelliten-Systeme für eine europäische Süßwassermuschelart (Ordnung Unionoida) darstellen. Die entwickelten Markersysteme weisen eine hohe Variabilität bezüglich ihrer Allelzahlen und Heterozygotiegrade auf und sind ideal geeignet, um die neutrale genetische Divergenz und Diversität der derzeitigen Populationsstruktur von Perlmuschelbeständen zu beschreiben.

Die genetische Diversität und Differenzierung der letzten und wichtigsten mitteleuropäischen Perlmuschelpopulationen aus den Einzugsgebieten von Elbe, Donau, Rhein, Maas und Weser wurde erfasst, um genetische Einheiten („Conservation Units“) zu definieren, prioritäre Populationen für den Artenschutz zu identifizieren und auf genetischer Basis Artenschutzmaßnahmen für freilebende Populationen und für Nachzuchtmaßnahmen abzuleiten. Die mitteleuropäischen Perlmuschelpopulationen sind stark fragmentiert und zeigen große Unterschiede in ihrer genetischen Variabilität. Dies lässt sich auf historische, demographische und anthropogene Effekte zurückführen.

Für die DNA-Untersuchungen wurden nicht-destruktive Probenahmemethoden (Totfunde und Hämolymph-Entnahme) angewandt, die keinen nachteiligen Einfluss auf die bestehenden Populationen haben. Darüber hinaus wird die Analyse von Schalen-DNA demonstriert und deren Potenzial für DNA-basierende Untersuchungen, notwendige Vorsichtsmaßnahmen und Grenzen dieser Methoden zur Vermeidung fehlerhafter Ergebnisse diskutiert.

Diese Arbeit belegt darüber hinaus die Möglichkeit, Jahresschichten der Carbonate von Perlmuschelschalen zu trennen und als Langzeitarchiv über einen Zeitraum von bis zu 100 Jahren zu analysieren. Untersuchungen der Signaturen stabiler Isotope in den Wachstumsschichten der inneren Perlmutter- und der äußeren Prismenschicht zeigen, dass die $\delta^{13}C$ Signaturen im Schalencarbonat Marker für individuelle metabolische Signale darstellen,

die mehrjährige Trends aufweisen. Zur Schalenaragonitbildung trägt ein hoher Anteil von respiratorischem CO_2 (aus Umweltrespiration und Respiration der Muschel) bei. In Verbindung mit der Analyse von $\delta^{15}N$ Signaturen von Muschelgeweben und potenziellen Nahrungsquellen können die Trophieebene der Muscheln und die Herkunft der Muschelnahrung bestimmt werden.

Durch Untersuchungen zum Status der Wirtsfischbestände und der Fischartendiversität in europäischen Perlmuschelgewässern wurden typische Fischbiozönosen in diesen Gewässerbereichen charakterisiert und gezeigt, dass ein Mangel an Wirtsfischen offenbar nur in bestimmten Regionen für die Reproduktion der Perlmuscheln limitierend ist. Intakte und funktionale Perlmuschelpopulationen wurden unter extrem oligotrophen Verhältnissen nachgewiesen, die niedrigere Wirtsfischdichten und Biomassen als die beeinträchtigten mitteleuropäischen Populationen ohne Jungmuschelnachwuchs aufweisen. Die Auswirkungen von Besatzmaßnahmen mit glochidieninfizierten Wirtsfischen als Artenschutzmaßnahme wurden diskutiert.

Aufgrund der weiten geographischen Verbreitung der Perlmuschel, ihres komplexen Entwicklungszyklus und der außergewöhnlichen Fortpflanzungsstrategie kann das globale Phänomen des starken Bestandesrückgangs in verschiedenen Regionen unterschiedliche und multiple Gründe haben. Artenschutzstrategien für die Flussperlmuschel profitieren von einem kombinierten Ansatz aus Molekulargenetik und Ökologie, der einerseits ein Maximum der Biodiversität und des evolutionären Potenzials der Art sichert und der andererseits die Habitatansprüche der Art identifiziert und ein geeignetes Habitat für alle Phasen des Entwicklungszyklus wiederherstellt. Artenschutzstrategien für die Flussperlmuschel stellen ein wichtiges Schlüsselbeispiel für die Entwicklung von Schutzstrategien für andere aquatische Lebewesen und für die ökosystemare Funktionalität, von der sie abhängen, dar.

Grundsätzlich sollten Schutzbemühungen vom statischen Schutz einzelner Arten und der Erhaltung eines bestimmten Verteilungsmusters hin zum nachhaltigen Schutz der Prozesse in Ökosystemen (Prozessschutz) entwickelt werden.

11 References

Altmüller R, Dettmer R (2001) Erhaltungsmaßnahmen für die Flussperlmuschel in der Lüneburger Heide: Ein Situationsbericht; In: *The freshwater pearl mussel in Europe: Population status and conservation strategies*; WWA Hof, Albert-Ludwigs-Universität Freiburg (eds); 146-156

Alvarez-Claudio C, Garcia-Roves P, Ocharan R, Cabal JA, Ocharan FJ, Alvarez MA (2000) A new record of the freshwater pearl mussel *Margaritifera margaritifera* L (Bivalvia: Unionoida) from the river Narcea (Asturias, north-western Spain); *Aquatic Conservation: Marine and Freshwater Ecosystems* **10**; 93-102

Araujo R, Ramos A (2000) Action plan for *Margaritifera margaritifera* in Europe; Convention on the conservation of European Wildlife and natural habitats, Strasbourg, 41pp., available at http://www.nature.coe.int/CP/tpvs10e.htm

Auerswald K (1990) Einfluß von Boden-pH und Bodenwasserhaushalt auf die Gewässerversauerung im ostbayerischen Grundgebirge; *Laufener Seminarbeiträge* **4/90**; 23-29

Bauer G (1986) The status of the freshwater pearl mussel in the south of its European range; *Biological Conservation* **37**; 1-9

Bauer G (1987a) Reproductive strategy of the freshwater pearl mussel *Margaritifera margaritifera*; *Journal of Animal Ecology* **56;** 691-704

Bauer G (1987b) The parasitic stage of the freshwater pearl mussel (*Margaritifera margaritifera* L.) III. Host relationships; *Archiv für Hydrobiologie* **76**, 413-423

Bauer G (1987c) The parasitic stage of the freshwater pearl mussel (*Margaritifera margaritifera* L.) II. Susceptibility of brown trout; *Archiv für Hydrobiologie* **76**; 403-412

Bauer G (1988) Threats to the freshwater pearl mussel *Margaritifera margaritifera* in central Europe; *Biological Conservation* **45**; 239-253

Bauer G (1991) Plasticity in life history traits of the freshwater pearl mussel; In: Seitz A and Loeschke W (eds); *Species conservation: A population biological approach*; Birkhauser Verlag Basel; 103-120

Bauer G (1992) Variation in the life span and size of the freshwater pearl mussel; *Journal of Animal Ecology* **61**; 425-436

Bauer G (1994) The adaptive value of offspring size among freshwater mussels (Bivalvia: Unionoidea); *Journal of Animal Ecology* **63;** 933-944

Bauer G (1998) Allocation policy of female freshwater pearl mussels; *Oecologia* **117;** 90-94

Bauer G, Hochwald S, Silkenat W (1991) Spatial distribution of freshwater mussels: The role of host fish and metabolic rate; *Freshwater Biology* **26**; 377-386

Bauer G, Vogel C (1987) The parasitic stage of the freshwater pearl mussel (*Margaritifera margaritifera*) I. Host relationships; *Archiv für Hydrobiologie* **76**; 413-423

Bauer G, Wächtler K (2001) Ecology and Evolution of the Freshwater Mussels Unionoida; *Ecological Studies* **145**; Springer Verlag, Heidelberg

Bellemain E, Taberlet P (2004) Improved non-invasive genotyping method: application to brown bear (*Ursus arctos*) faeces; *Molecular Ecology Notes* **4**; 519-522

Bernatchez L (2001) The evolutionary history of brown trout (*Salmo trutta* L.) inferred from phylogeographic, nested clade, and mismatch analyses of mitochondrial DNA variation; *Evolution* **55**; 351-379

Bogan AE (1993) Freshwater bivalve extinctions (Mollusca: Unionoida): A search for causes; *American Zoologist* **33**; 599-609

Bogan AE (1998) Freshwater molluscan conservation in North America: Problems and practises; *Journal of Conchology Special Publication* **2**; 223-230

Bond WJ (1993) Keystone species; In: Schulze ED and Mooney HA (eds): Ecosystem Function and Biodiversity; Springer Verlag, Berlin; 237-253

Borchardt T (1985) Relationships between carbon and cadmium uptake in *Mytilus edulis*; *Marine Biology.* **85**; 233-244

Boss KJ (1982) Mollusca; In: Parker SP (ed) Synopsis and classification of living organisms, vol 1. McGraw-Hill, New York; pp. 945-1116

Bouchet P, Falkner G, Seddon MB (1999) Lists of protected land and freshwater molluscs in the Bern Convention and European Habitats Directive: are they relevant to conservation; *Biological Conservation* **90**; 21-31

Bowen BW (1999) Preserving genes, species, or ecosystems? Healing the fractured foundations of conservation policy; *Molecular Ecology* **8**; S5-S10

Buddensiek V (1995) The culture of juvenile pearl mussels *Margaritifera margaritifera* in cages: a contribution to conservation programmes and the knowledge of habitat requirements; *Biological Conservation* **99**, 183-190

Buddensiek V, Engel H, Fleischauer-Rossing S, Wächtler K (1993) Studies on the chemistry of interstitial water taken from defined horizons in the fine sediments of bivalve habitats in several North German lowland waters II: microhabitats of *Margaritifera margaritifera L.*; *Archiv für Hydrobiologie* **127**; 151-166

Carell B, Forberg S, Grundelius E, Henrikson L, Johnels A, Lindh U, Mutvei H, Olsson M, Svardstrom K, Westermark T (1987) Can mussel shells reveal environmental history? *Ambio* **16**; 2-10

Chesney HCG, Oliver PG, Davis GM (1993) *Margaritifera durrovensis* Phillips, 1928: Taxonomic status, ecology and conservation; *Journal of Conchology* **34**; 267-299

Chesney HCG, Oliver PG (1998) Conservation issues for Margaritiferidae in the British Isles and Western Europe; *Journal of Conchology, Special Publication* **2**; 231-242

Ciofi C, Bruford MW (1999) Genetic structure and gene flow among Komodo dragon populations inferred by microsatellite loci analysis; *Molecular Ecology* **8**, 17-30
[2MOD program available at: http://www.rubic.rdg.ac.uk/~mab/software.html]

Clark JA, May RM (2002) Taxonomic bias in conservation research; *Science* **297**; 191-192

Committee on Scientific Issues in the Endangered Species Act (CSIESA) (1995) Science and the Endangered Species Act. National Academy Press, Washington, D.C.

Cornuet JM, Luikart G (1996) Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data; *Genetics* **144**; 2001-2014

Cornuet JM, Piry S, Luikart G, Estoup A, Solignac M (1999) New methods employing multilocus genotypes to select or exclude populations as origins of individuals; *Genetics* **153**; 1989-2000

Cosgrove PJ, Young MR, Hastie LC, Gaywood M, Boon PJ (2000) The status of the freshwater pearl mussel *Margaritifera margaritifera* Linn. in Scotland; *Aquatic Conservation: Marine and Freshwater Ecosystems* **10**; 197-208

Craig H (1953) The geochemistry of the stable carbon isotopes; *Geochimica et Cosmochimica Acta* **3**; 53-65

Crandall KA, Bininda-Emonds ORP, Mace GM, Wayne RK (2000) Considering evolutionary processes in conservation biology; *Trends in Ecology and Evolution* **15**; 290-295

Cunjak RA, McGladdery SE (1991) The parasite-host relationship of glochidia (Mollusca: Margaritiferidae) on the gills of young-of-the-year Atlantic salmon (Salmo salar); *Canadian Journal of Zoology* **69**; 353-358

Davis GM, Fuller SLH (1981) Genetic relationships among recent Unionacea (Bivalvia) of North America; *Malacologia* **20**; 217-253

De Niro MJ, Epstein S (1981) Influence of diet on the distribution of nitrogen isotopes in animals; *Geochimica et Cosmochimica Acta* **45**; 341-351

Dettman DL, Lohmann KC (1993) Seasonal changes in paleogene surface water $\delta^{18}O$: Fresh-water bivalves of western North America; In *Climate Change in Continental Isotopic Records. (ed.* Swart, P. K., *et al.*), Vol. **78** ; pp. 153-163; AGU Monograph

Dettman DL, Reische AK, Lohmann KC (1999) Controls on the stable isotope composition of seasonal growth bands in aragonitic fresh-water bivalves (unionidae); *Geochimica et Cosmochimica Acta* **63**; 1049-1057

Donner J, Nord AG (1986) Carbon and oxygen stable isotope values in shells of *Mytilus edulis* and *Modiolus modiolus* from Holocene raised beaches at the outer coast of the Varanger peninsula, North Norway; *Palaeogeography Palaeoclimatology Palaeoecology* **56**; 35-50

Eackles MS, King TL (2002) Isolation and characterisation of microsatellite loci in *Lampsilis abrupta* (Bivalvia: Unionidae) and cross-species amplification within the genus; *Molecular Ecology Notes* **2**; 559-562

Egglishaw HJ, Shackley PE (1977) Growth, survival and reproduction of juvenile salmon and trout in a Scottish stream, 1966-75; *Journal of Fish Biology* **11**; 647-672.

Eklöv AG, Greenberg LA (1998) Effect of artificial instream cover on density of 0+ brown trout; *Fisheries Management & Ecology* **5**; 45-53

Engel H, Wächtler K (1989) Some peculiaries in the developmental biology of two forms of the freshwater bivalve Unio crassus in northern Germany; *Archiv für Hydrobiologie* **115**; 441-450

Epstein S, Buchsbaum R, Lowenstam HA and Urey HC (1953) Revised carbonate-water isotopic temperature scale; *Geological Society of America Bulletin* **64**; 1315-1325

Estoup A, Solignac M, Harry M, Cornuet JM (1993) Characterisation of $(GT)_n$ and $(CT)_n$ microsatellites in two insect species: *Apis mellifera* and *Bombus terrestris*; *Nucleic Acids Research* **21**; 1427-1431

Excoffier L, Smouse PE, Quattro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data; *Genetics* **131**; 479-491

Friedli H, Lötscher H, Oeschger H, Siegenthaler U, Staffer B (1986) Ice core record of the $^{13}C/^{12}C$ ratio of atmospheric CO_2 in the past two centuries; *Nature* **324**; 237-238

Fritz P, Poplawski S (1974) ^{18}O and ^{13}C in shells of freshwater molluscs and their environments; *Earth and Planetary Science Letters* **24**; 91-98

Fry B, Allen YC (2003) Stable isotopes in zebra mussels as bioindicators of river-watershed linkages; *River Research and Applications* **19**; 683-696

Gaston KJ, May RM (1992) The taxonomy of taxonomists; *Nature* **356**; 281-282

Geist J (1999a) Schadwirkungen von Feinsedimenten in Flussperlmuschelgewässern; *die Flussmeister; Zeitschrift für Wasserwirtschaft*; 43-46

Geist J (1999b) Ist die Flussperlmuschel noch zu retten? Geoökologische Aspekte im Gewässerschutz; *junge wissenschaft* **55**; 18-24

Geist J (2002) Entwicklung molekulargenetischer Marker bei der Flussperlmuschel (*Margaritifera margaritifera* L.); Diplomarbeit im Fach Biotechnologie der Tiere an der Technischen Universität München

Geist J, Rottmann O, Schröder W, Kühn R (2003) Development of microsatellite markers for the endangered freshwater pearl mussel *Margaritifera margaritifera* L. (Bivalvia: Unionoidea); *Molecular Ecology Notes* **3**; 444-446

Geist J, Schmidt C (2004) Besatzmaßnahmen mit Muscheln; *Bayerns Fischerei und Gewässer No. 3/2004;* I-IV

Geist J, Kuehn R (2005) Genetic diversity and differentiation of central European freshwater pearl mussel (*Margaritifera margaritifera* L.) populations: implications for conservation and management; *Molecular Ecology* **14**; 425-239

Goudet J (2001) FSTAT, version 2.9.3: a program to estimate and test gene diversities and fixation indices; [Available at http://www.unil.ch/izea/softwares/fstat.html]

Guo SW, Thompson EA (1992) Performing the exact test of Hardy-Weinberg proportion for multiple alleles; *Biometric* **48**; 361-372

Haas F (1969a) Superfamilia Unionacea; In Das Tierreich, vol. 88 Walter de Gruyter and Co, Berlin

Haas F (1969b) Superfamily Unionacea Fleming 1828; In: Moore RC (ed) Treatise of invertebrate paleontology, Part Mollusca 6, vol 1. Bivalvia. Geological Society of America, Boulder, Colorado; University of Kansas press, Lawrence, Kansas; pp N411-N414

Haldane JBS (1954) An exact test for randomness of mating; *Journal of Genetics* **52**; 631-635

Hänfling B, Brandl R (1998) Genetic variability, population size and isolation of distinct populations in the freshwater fish *Cottus gobio* L.; *Molecular Ecology* **7**; 1625-1632

Hantke R (1993) Flussgeschichte Mitteleuropas; Ferdinand Enke Verlag, Stuttgart

Harris D, Horwath WR, van Kessel C (2001) Acid fumigation of soils to remove carbonates prior to total organic carbon or carbon-13 isotopic analysis; *Soil Science Society of American Journal* **65**; 1853–1856

Hastie LC, Boon PJ (2001) Does electric fishing harm the freshwater pearl mussels? *Aquatic Conservation: Marine and Freshwater Ecosystems* **11**; 149-152

Hastie LC, Cosgrove PJ (2001) The decline of migratory salmonids: a new threat to pearl mussels in Scotland; *Freshwater Forum* **15**; 85-96

Hastie LC, Cosgrove PJ. (2002) Intensive searching for mussels in a fast-flowing river: an estimation of sampling bias; *Journal of Conchology* **37**; 309-316

Hastie LC, Young MR, Boon PJ (2000) Growth characteristics of freshwater pearl mussels, *Margaritifera margaritifera* (L.); *Freshwater Biology* **43**; 243-256

Hastie LC, Young MR (2003a) Conservation of the freshwater pearl mussel 1. Captive breeding techniques; *Conserving Natura 2000 Rivers Conservation Techniques Series No.2*. English Nature, Peterborough

Hastie LC, Young MR (2003b) Conservation of the freshwater pearl mussel 2. relationship with salmonids; *Conserving Natura 2000 Rivers Conservation Techniques Series No.3;* English Nature, Peterborough; 44pp

Heard WH, Gluckert RH (1970) A re-evaluation of the recent Unionacea (Pelecypoda) of North America; *Malacologia* **10**; 333-335

Hedrick PW (2004) Recent developments in conservation genetics; *Forest Ecology and Management* **197**; 3-19

Hochwald S (1997) Das Beziehungsgefüge innerhalb der Größenwachstums- und Fortpflanzungsparameter bayerischer Bachmuschelpopulationen (*Unio crassus* Phil 1788) und dessen Abhängigkeit von Umweltparametern. PhD thesis, University of Bayreuth; *Bayreuther Forum Ökologie*, Band **50**; 166pp

Hofreiter M, Serre D, Poinar HN, Kuch M, Pääbo S (2001a) Ancient DNA; *Nature Reviews Genetics* **2**; 353-359

Hofreiter M, Jaenicke V, Serre D, Haeseler Av A, Pääbo S (2001b) DNA sequences from multiple amplifications reveal artifacts induced by cytosine deamination in ancient DNA; *Nucleic Acids Research* **29**; 4793-4799

Huff SW, Campbell D, Gustafson DL, Lydeard C, Altaba C, Giribet G (2004) Investigations into the phylogenetic relationships of freshwater pearl mussels (Bivalvia: Margaritiferidae) based on molecular data: implications for their taxonomy and biogeography; *Journal of Molluscan Studies* **70**; 379-388

Israel W (1913) Biologie der europäischen Süßwassermuscheln; K.G. Lutz Verlag; Stuttgart; 44-47

Jansen W, Bauer G, Zahner-Meike E (2001) Glochidial mortality in freshwater mussels; In: Bauer G and Wachtler K (eds) Ecology and Evolutionary Biology of the freshwater mussels Unionoidea; *Ecological Studies* **145**, Springer Verlag, Heidelberg; 185-211

Johnson RI (1970) Systematics and zoogeography of Plagiola (= Dysnomia = Epioblasma), an almost extinct genus of freshwater mussels (Bivalvia: Unionidae) from middle North America; *Bulletin of the Museum of Comparative Zoology* **148**; 239-392

Jungbluth JR, Coomans HE, Groh H (1985) Bibliographie der Flussperlmuschel *Margaritifera margaritifera* (Linn. 1785); Verslagenen Technische Gegevens No 41; Institut voor Taxonomische Zoologie, Universiteit Amsterdam

Kaandorp RJG, Vonhof HB, Del Busto C, Wesselingh FP, Ganssen GM, Marmól AE, Pittman LR, van Hinte JE (2003) Seasonal stable isotope variations of the modern Amazonian freshwater bivalve *Anodonitites trapesialis*; *Paleogeography Paleoclimatology Palaeoecology* **194**; 339-354

Keeling CD, Whorf TP, Wahlen M, van der Plicht J (1995) Interannual extremes in the rate of rise of atmospheric carbon-dioxide since 1980; *Nature* **375**; 666-670

Keith ML, Anderson GM, Eichler R (1964) Carbon and oxygen isotopic composition of mollusk shells from marine and fresh-water environments; *Geochimica et Cosmochimica Acta* **28**; 1757-1786

Kimura M, Crow JF (1963) On the maximum avoidance of inbreeding; *Genetical Research* **4**; 399-415

Klein RT, Lohmann KC, Thayer CW (1996) Sr/Ca and $^{13}C/^{12}C$ ratios in skeletal calcite of *Mytilus trossulus*: Covariation with metabolic rate, salinity, and carbon isotopic composition of seawater; *Geochimica et Cosmochimica Acta* **60**; 4207-4221

Kumar S, Tamura K, Nei M (1993) *MEGA version 1.0 : Molecular Evolutionary Genetic Analysis*; Pennsylvania State University, Pennsylvania

Larsen BM (2001) Overvåking av elvemusling *Margaritifera margaritifera* i Norge; *Årsrapport 2000. – NINA Oppdragsmelding* 725; 1-43

Lenormand T (2002) Gene flow and the limits to natural selection; *Trends in Ecology & Evolution* **17**; 183-189

Lindh U, Mutvei H, Sunde T, Westermark T (1988) Environmental history told by mussel shells; *Nuclear Instruments and Methods in Physics Research, Section B*. **30**; 388-392

Luck GW, Daily GC, Ehrlich PR (2003) Population diversity and ecosystem services; *Trends in Ecology and Evolution* **18**; 331-336

Lydeard C, Cowie RH, Ponder WF, Bogan AE, Bouchet P, Clark AS, Cummings KS, Frest TJ, Gargominy O, Herbert DG, Hershler R, Perez KE, Roth B, Seddon M, Strong EE, Thompson FG (2004) The global decline of nonmarine molluscs; *Bioscience* **54**; 321-329

Lydeard C, Lindberg DR (2003) Molecular Systematics and Phylogeography of Molluscs; Smithsonian Series in Comparative Evolutionary Biology (ed. Lydeard C, Lindberg DR), Smithonian Books Washington and London

Machordom A, Araujo R, Erpenbeck D, Ramos MA (2003) Phylogeography and conservation genetics of endangered European Margaritiferidae (Bivalvia: Unionoidea); *Biological Journal of the Linnean Society* **78**; 235-252

Manel S, Schwartz MK, Luikart G, Taberlet P (2003) Landscape genetics: combining landscape ecology and population genetics; *Trends in Ecology and Evolution* **18**; 189-197

McConnaughey TA (1989) ^{13}C and ^{18}O isotopic disequilibrium in biological carbonates: I. Patterns; *Geochimica et Cosmochimica Acta* **53**; 151-162

McConnaughey TA (2003) Sub-equilibrium oxygen-18 and carbon-13 levels in biological carbonates: carbonate and kinetic models; *Coral Reefs* **22**; 316-327

McConnaughey TA, Burdett J, Whelan JF, Paull CK (1997) Carbon isotopes in biological carbonates: Respiration and photosynthesis; *Geochimica et Cosmochimica Acta* **61**; 611-622

Meißner (1912) Die Perlmuscheln in Oberfranken; *II. Bericht der Naturwissenschaftlichen Gesellschaft Bayreuth für die Zeit von Herbst 1911 bis Frühjahr 1914*, Bayreuth 1914

Menge BA, Berlow EL, Blanchette CA, Navarette SA, Yamanda SB (1994) The keystone species concept: variation in interaction strength in a rocky intertidal habitat; *Ecological Monographs* **64**; 249-286

Miguel ES, Monserrat S, Fernández C, Amaro R, Hermida M, Ondina P, Altaba CR (2004) Growth models and longevity of pearl mussels (*Margaritifera margaritifera*) in Spain; *Canadian Journal of Zoology – Review Canadienne de Zoologie* **82**; 1370-1379

Mook WG (1971) Paleotemperatures and chlorinities from stable carbon and oxygen isotopes in shell carbonate; *Palaeogeography Palaeoclimatology Palaeoecology* **9**; 245-263

Moorkens EA, Costello MJ (1994) Imminent extenuation of the Nore freshwater pearl mussel *Margaritifera durrovensis* Phillips: a species unique to Ireland; *Aquatic Conservation: Marine and Freshwater Ecosystems* **4**; 363-365

Morales JJ, Negro AI, Lizana M, Martinez A, Palacios J (2004) Preliminary study of the endangered populations of pearl mussels *Margaritifera margaritifera* (L.) in the river Tera (north-west Spain): habitat analysis and management considerations; *Aquatic Conservation: Marine and Freshwater Ecosystems* **14**;587-596

Morin PA, Chambers KE, Boesch C, Vigilant L (2001) Quantitative polymerise chain reaction analysis of DNA from non-invasive samples for accurate microsatellite genotyping of wild chimpanzees (*Pan troglodytes verus*); *Molecular Ecology* **10**; 1835-1844

Moritz C (1994) Defining evolutionary significant units for conservation; *Trends in Ecology and Evolution* **9**; 373-375

Moritz C (2002) Strategies to protect biological diversity and the evolutionary processes that sustain it; *Systematic Biology* **51**; 238-254

Moritz C, Richardson KS, Ferrier S, Monteith GB, Stanisic J, Williams SE, Whiffin T (2001) Biogeographical concordance and efficiency of taxon indicators for establishing conservation priority in a tropical rainforest biota; *Proceedings of the Royal Society of London B* **268**; 1875-1881

Mutvei H, Westermark T (2001) How environmental information can be obtained from naiad shells; In: Bauer G and Wächtler K (eds) Ecology and Evolutionary Biology of the freshwater mussels Unionoidea. *Ecological Studies* **145**; Springer Verlag, Heidelberg; 367-379

Myers N (2003) Biodiversity hotspots revisited; *BioScience* **53**; 916-917

Nagel KO, Badino G, Celebrano G (1998) Systematics of European Naiades (Bivalvia: Margaritiferidae and Unionidae): A review and some new aspects; Bivalvia I; *Malacological Review* **7**; 83-104

Nagel KO, Badino G (2001) Population Genetics and Systematics of European Unionoidea; In: Bauer G, Wächtler K (eds) Ecology and Evolution of the freshwater mussels Unionoidea; *Ecological Studies* **145**; Springer Verlag, Heidelberg; 51-80

Nei M, Tajima F, Tateno Y (1983) Accuracy of genetic distances and phylogenetic trees from molecular data; *Journal of Molecular Evolution* **19**; 153-170

Neves RJ, Bogan AE, Williams JD, Ahlstedt SA, Hartfield PW (1997) Status of aquatic molluscs in the southeastern United States: A downward spiral of diversity; In: Aquatic fauna in peril: The southeastern perspective (GW Benz and DE Collins, eds) Special Publication 1, Southeast Aquatic Research Institute, Lenz Design and Communications, Decatur, GA

Nielsen DR, Wendroth O (2003) *Spatial and temporal statistics.* 1-398; Reiskirchen, Catena Verlag. GeoEcology.

Nunney L, Campell KA (1993) Assessing minimum viable population size: demography meets population genetics; *Trends in Ecology and Evolution* **8**; 234-239

Nyström J, Lindh U, Dunca E, Mutvei H (1995) A study of *Margaritifera margaritifera* shells from the river Paulistromsan, S. Sweden; *Nuclear Instruments and Methods in Physics Research, Section B* **104**; 612-618

Ota T (1993) *DISPAN: Genetic distance and phylogenetic analyses software*; Pennsylvania. Pennsylvania State University

Parodiz JJ, Bonetto AA (1963) Taxonomy and zoogeographic relationships of the South American naiads (Pelecypoda: Unionacea and Mutelacea); *Malacologia* **1**; 179-213

Petit RJ, Mousadik El, Pons O (1998) Identifying populations for conservation on the basis of genetic markers; *Conservation Biology* **12**; 844-855

Piasentier E, Valusso R, Camin F, Versini G (2003) Stable isotope ratio analysis for authentication of lamb meat; *Meat Science* **64**; 239-247

Piry S, Cornuet JM (1999) *GENECLASS: A Program for Assignation and Exclusion using Molecular Markers*; France: URLB/INRA
[Available at: http://www.ensam.inra.fr/URLB/geneclass/geneclass.html]

Piry S, Luikart G, Cornuet JM (1999) BOTTLENECK: a computer programme for detecting recent reductions in the effective population size using allele frequency data; *Journal of Heredity* **90**; 502-503
[Available at: http://www.ensam.inra.fr/URLB/bottleneck/bottleneck.html]

Ponder WF, Lunney D (1999) The other 99% : The conservation and biodiversity of invertebrates; Mosman (Australia): Royal Society of New South Wales

Ponsard S, Averbuch P (1999) Should growing and adult animals fed on the same diet show different $\delta^{15}N$ values? *Rapid Communications in Mass Spectrometry* **13**; 1305-1310

Raymond M, Rousset F (1995a) *GENEPOP version 3.3*: population genetics software for exact tests and ecumenicism; *Journal of Heredity* **86**; 248-249
[Available at: ftp://ftp.cefe.cnrs-mop.fr/pub/pc/msdos/genepop]

Raymond M, Rousset F (1995b) An exact test for population differentiation; *Evolution* **49**; 1280-1283

Reed DH, Frankham R (2003) Correlation between fitness and genetic diversity; *Conservation Biology* **17**; 230-237

Reis J (2003) The freshwater pearl mussel [*Margaritifera margaritifera* (L.)] (Bivalvia, Unionoida) rediscovered in Portugal and threats to ist survival; *Biological Conservation* **114**; 447-452

Ricciardi A, Rasmussen JB (1999) Extinction rates of North American fauna; *Conservation Biology* **13**; 1220-1222

Rice WR (1989) Analyzing tables of statistical tests; *Evolution* **43**; 223-225

Roe KJ, Hoeh WR (2003) Systematics of freshwater mussels (Bivalvia: Unionoida). In: *Molecular Systematics and Phylogeography of Molluscs; Smithsonian Series in Comparative Evolutionary Biology* (ed. Lydeard C, Lindberg DR), Smithonian Books Washington and London; pp. 91-122

Romanek CS, Grossman EL, Morse JW (1992) Carbon isotopic fractionation in synthetic aragonite and calcite – effects of temperature and precipitation rate; *Geochimica et Cosmochimica Acta* **56**; 419-430

Ronce O, Kirkpatrick M (2001) The sources become sinks: migration meltdown in in heterogenous habitats; *Evolution: International Journal of Organic Evolution* **55**; 1520-1531

Rossmann A (2001) Determination of stable isotope ratios in food analysis; *Food Reviews International* **17**; 347-381

Rozen S, Skaletsky HJ (1998) Primer3. Code available at: http//www-genome.wi.mit.edu/genome_software/other/primer3.html

Rudzite M (2004) Distribution of the freshwater pearl mussel *Margaritifera margaritifera* (Linnaeus 1758) in Latvia in relation to water quality; *Acta Universitatis Latviensis, Biology* **676**; 79-85

Sachteleben J, Schmidt C, Vandré R, Wenz G (2004) Leitfaden Flussperlmuschelschutz; Bayerisches Landesamt für Umweltschutz (ed); Augsburg

Sambrook J, Fritsch EF, Maniatis T (1991) Molecular Cloning: A Laboratory Manual, 3rd edn. Cold Spring Harbour Laboratory Press, New York

Scherf H (1980) Stirbt die Flussperlmuschel in Europa aus; *Naturwissenschaftliche Rundschau* **33**; 342-343

Schmidt C, Vandré R, Wenz G (2000) Natürliche und künstliche Infektion von Bachforellen mit Flußperlmuschelglochidien im Wolfertsrieder Bach, Landkreis Regen; Schlussbericht für das Bayerische LfU, Augsburg

Schmidt C, Wenz G (2000) Kontinuierliche Überwachung der Flussperlmuschel in Bayern und Maßnahmen zur Bestandsstützung; *The freshwater pearl mussel in Europe: Population status and conservation strategies*; WWA Hof, Albert-Ludwigs-Universität Freiburg (eds); 92-101

Schmidt C, Wenz G (2001) Monitoring-Programm für ausgewählte Bestände der Flussperlmuschel (*Margaritifera margaritifera* L. 1758) als Datengrundlage für die Erfolgskontrolle von Schutzprojekten im Rahmen des Artenhilfsprogramms; BayLfU **156**; 373-393

Schneider S, Roessli D, Excoffier L (2000) *ARLEQUIN: A software for Population Genetic Data Analysis, version 2.000*; Genetics and Biometry Laboratory, University of Geneva, Switzerland. [ARLEQUIN v. 2.000 available at: http://lgb.unige.ch/arlequin/]

Schreckenbach K (1995) Untersuchungen zum Ernährungszustand von Flußperl- und Teichmuscheln (*Margaritifera margaritifera* und *Anodonta anatina*); *Lindberger Hefte* **5**; 84-97

Siegele R, Orlic I, Cohen DD, Markich SJ, Jeffree RA (2001) Manganese profiles in freshwater mussel shells; *Nuclear Instruments and Methods in Physics Research, Section B* **181**; 593-597

Simberloff D (1998) Flagships, umbrellas, and keystones: Is single-species management passé in the landscape era? *Biological Conservation* **83**; 247-257

Simberloff D, Abele LG (1982) Refuge design and island biogeographic theory: effects of fragmentation; *American Naturalist* **120**; 41-50

Skinner A, Young M, Hastie L (2003) Ecology of the freshwater pearl mussel; *Conserving Natura 2000 Rivers Ecology Series No.2* English Nature, Peterborough, 16pp

Smith DG (2001) Systematics and distribution of the recent Margaritiferidae; In: Bauer G, Wächtler K (eds) Ecology and evolution of the freshwater mussels Unionoida; Springer Verlag, Heidelberg; 33-49

Smith DG, Wall WP (1985) The Margaritiferidae reinstated: a reply to Davis and Fuller (1981) ´Genetic relationships among recent Unionacea (Bivalvia) of North America´; *Occasional Papers Molluscs* **64**; 321-332

Soulé ME (1987) Viable populations for conservation; Cambridge University Press

Strayer DL, Downing JA, Haag WR, King TL, Layzer JB, Newton TJ, Nichols SJ (2004) Changing perspectives on pearly mussels, North America´s most imperiled animals; *BioScience* **54**; 429-439

Taberlet P, Waits LP, Luikart G (1999) Noninvasive genetic sampling: look before you leap; *Trends in Ecology and Evolution* **14**; 323-327

Tanaka N, Monaghan MC, Rye DM (1986) Contribution of metabolic carbon to mollusc and barnacle shell carbonate; *Nature* **320**; 520-523

Templeton AR (1986) Coadaption and outbreeding depression; In: *Conservation Biology: The science of scarcity and diversity* (ed. Soulé ME); Sinauer Massachusetts, pp. 105-116

Tripati A, Zachos J, Marincovich L, Bice K (2001) Late Paleocene Arctic coastal climate inferred from molluscan stable and radiogenic isotope ratios; *Palaeogeography Palaeoclimatology Palaeoecology* **170**; 101-113

Valovirta I (1990) Conservation of *Margaritifera margaritifera* in Finland. Colloquium of the Bern Convention Invertebrates and their Conservation; *Council of Europe, Strasbourg*; T-PVS 34; 59-63

Vander Putten E, Dehairs F, Keppens E, Baeyens W (2000) High resolution distribution of trace elements in the calcite shell layer of modern *Mytilus edulis*: Environmental and biological controls; *Geochimica et Cosmochimica Acta* **64**; 997-1011

Vanderklift MA, Ponsard S (2003) Sources of variation in consumer-diet δN^{15} enrichment: a meta-analysis; *Oecologia* **136**; 169-182

Vaughn CC, Hakenkamp CC (2001) The functional role of burrowing bivalves in freshwater ecosystems (2001) *Freshwater Biology* **46**; 1431-1446

Veinott GI, Cornett RJ (1998) Carbon isotopic disequilibrium in the shell of the freshwater mussel *Elliptio complanata; Applied Geochemistry* **13**; 49-57

Velasco Marcos JC, Araujo Armero R, Bueno Hernandez R, Laguna Gumiel A (2002) Descubierta la población europea más merional conocida de la madreperla de río *Margaritifera margaritifera* L. (Bivalvia, Unionoida), en la península Ibérica (Río Águeda, Salamanca); Discovered the southernmost known european population of the freshwater pearl mussel *Margaritifera margaritifera* L. (Bivalvia, Unionoida), in the Iberian peninsula (Río Águeda, Salamanca); Sociedad Espanola de Malacología; Iberus **20**; 99-108

Wächtler K, Dettmer R, Buddensiek V (1987) Zur Situation der Flussperlmuschel (*Margaritifera margaritifera* (L.)) in Niedersachsen: Schwierigkeiten, eine bedrohte Art zu erhalten; Bericht der Naturhistorischen Gesellschaft Hannover **129**; 209-224

Wächtler K, Dreher-Mansur MC, Richter T (2001) Larval types and early postlarval biology in naiads (Unionoida); In: Bauer G, Wächtler K (eds) Ecology and Evolution of the freshwater mussels Unionoidea. *Ecological Studies* **145**; Springer Verlag, Heidelberg; 93-125

Watters GT (1994) Form and function of unionoidean shell sculpture and shape (Bivalvia); *American Malacological Bulletin* **11**; 1-20

Watters GT (2001) The evolution of the Unionacea in North America, and its implications for the worldwide fauna; In: Bauer G, Wächtler K (eds) Ecology and Evolution of the freshwater mussels Unionoidea; *Ecological Studies* **145**; Springer Verlag, Heidelberg; 281-307

Wefer G, Berger WH (1991) Isotope paleontology: growth and composition of extant calcareous species; *Marine Geology* **100**; 207-248

Weiss S, Schlötterer C, Waidbacher H, Jungwirth M (2001) Haplotype (mtDNA) diversity of brown trout *Salmo trutta* in tributaries of the Austrian Danube: massive introgression of Atlantic basin fish – by man or nature? *Molecular Ecology* **10**; 1241-1246

Williams JD, Warren ML, Cummings KS, Harris JL, Neves RJ (1993) Conservation status of the freshwater mussels of the United States and Canada; *Fisheries* **18**; 6-22

Wurster CM, Patterson WP, Cheatham MM (1999) Advances in micromilling techniques: a new apparatus for acquiring high-resolution oxygen and carbon stable isotope values and major/minor elemental ratios from accretionary carbonate; *Computers & Geosciences* **25**; 1159-1166

Young MR, Cosgrove PJ, Hastie LC (2001a) The extent of, and causes for, the decline of a highly threatened naiad: *Margaritifera margaritifera*; In: Bauer G, Wächtler K (eds) Ecology and Evolution of the freshwater mussels Unionoidea; *Ecological Studies* **145**; Springer Verlag, Heidelberg; 337-357

Young M, Hastie L, al-Mousawi B (2001b) What represents an ideal population profile for *Margaritifera margaritifera*; In: *The freshwater pearl mussel in Europe: Population status and conservation strategies*; WWA Hof, Albert-Ludwigs-Universität Freiburg (eds); 35-44

Young MR, Hastie LC, Cooksley SL (2003) Monitoring the freshwater pearl mussel, *Margaritifera margaritifera*; *Conserving Natura 2000 Rivers Monitoring Series No.2* English Nature, Peterborough

Young M, Purser GJ, Al-Mousawi B (1987) Infection and successful reinfection of brown trout [Salmo trutta (L.)] with glochidia of *Margaritifera margaritifera* (L.); *American Malacological Bulletin* **5**; 125-128

Young MR, Williams JC (1983) The status and conservation of freshwater pearl mussel *Margaritifera margaritifera* L. in Great Britain; *Biological Conservation* **25**; 35-52

Young MR, Williams JC (1984) The reproductive biology of the freshwater pearl mussel *Margaritifera margaritifera* (Linn.) in Scotland; I. Field Studies; *Archiv für Hydrobiologie* **99**; 405-422

Ziuganov V, Kaliuzhin S, Beletsky V, Popkovich E (2001) The pearl mussel-salmon community in the Varzuga river, northwest Russia: Problems and environmental impacts; In: Bauer G & Wächtler K (eds) Ecology and Evolution of the freshwater mussels Unionoida; *Ecological Studies* **145**; Springer Verlag, Heidelberg; 359-366

Ziuganov VV, Nezlin LP (1988) Evolutionary aspects of symbiosis of pearl mussels and salmonid fishes; In: The problems of macroevolution; Moscov; Nauka; 110-111

Ziuganov V, Zotin A, Nezlin L, Tretiakov V (1994) The freshwater pearl mussels and their relationships with salmonid fish; VNIRO, Russian Federal Institute of Fisheries and Oceanography, Moscow; 104pp

Zouros E, Foltz DW (1984) Possible explanations of heterozygote deficiency in bivalve molluscs; *Malacologia* **25**; 583-591