Host-parasite interactions between freshwater pearl mussels (*Margaritifera margaritifera*) and their salmonid hosts

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Thesis for the degree of Philosophiae Doctor (PhD)
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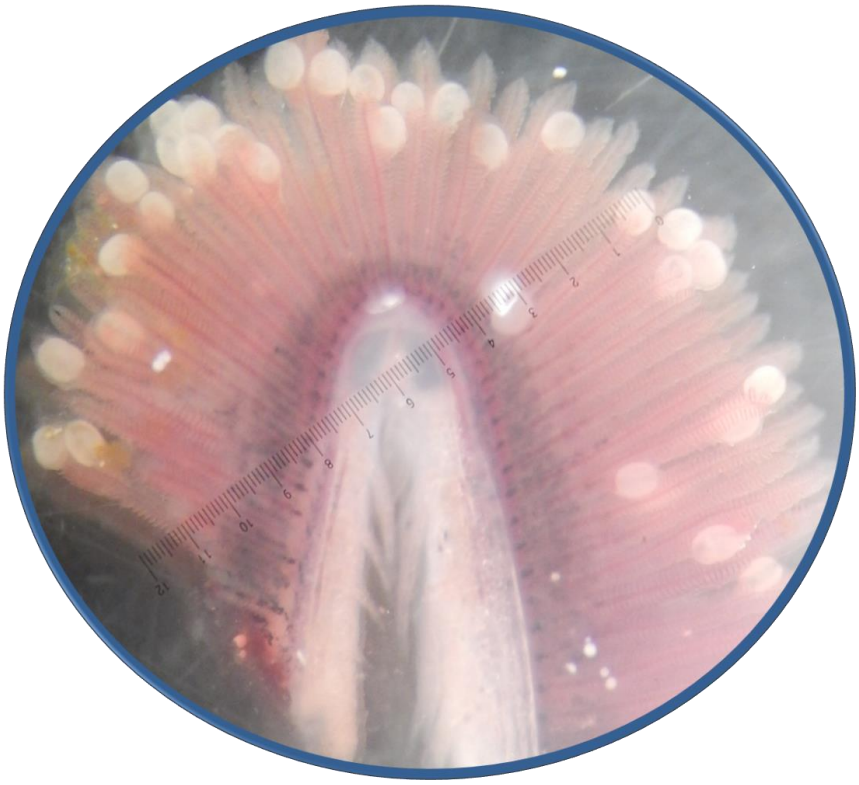

Margaritifera margaritifera on host gills

Scientific environment

This thesis was carried out at the Institute of Biology, Faculty of Mathematics and Natural Sciences, University of Bergen. The majority of the experimental work was performed at the freshwater rearing facilities in Austevoll. Genetic analyses of glochidia and juvenile mussels were performed at the Unit of Molecular Zoology at the Technische Universität München, and at the Norwegian Institute for Nature Research, respectively.

The thesis was supervised by Dr. Per Johan Jakobsen (University of Bergen), Dr. Juergen Geist (Technische Universität München) and Bjørn Mejdell Larsen (Norwegian Institute for Nature Research).

Dedication

I dedicate this thesis to Baba, my late grandfather, who was the rock in my life, who encouraged me to follow my dreams and believed that I could achieve them. I miss you and wish you were here today.

I also dedicate this work to Einar, now mum will finally be able to chillax and watch endless episodes of Full House, play many rounds of Jump in the ocean and take you swimming every weekend.

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Preface

This study was conducted to examine the interaction between freshwater pearl mussels (*Margaritifera margaritifera*) and their salmonid hosts. Host-parasite interactions, and the factors that influence them, were examined with the aim of contributing new knowledge that could be used to improve conservation strategies.

The first chapter of the thesis gives a general introduction to the species *M. margaritifera*, with background information on its life cycle, host-specificity, host-parasite interactions, threats and current status in Norway. In the following three chapters, specific research questions concerning host-parasite interactions are presented. Chapters 2 and 3 have been published as research papers, and are therefore written in the format required by the journals. In Chapter 5, the results of the research questions are reviewed in a general discussion, including their implications for future conservation efforts.

Acknowledgements

I have spent several incredible years studying and helping to conserve the freshwater pearl mussel, *Margaritifera margaritifera*. During these years I have had the opportunity to meet many researchers with a passion for freshwater bivalves, either at conferences or those who visited the rearing station at Austevoll. I have had the opportunity to collaborate with several research laboratories, and have made many good friends over these years. I would like to thank all the people who have contributed physically and mentally to this thesis. Without the help and support of my supervisors, colleagues, family and friends, this thesis would not have been possible.

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I am especially grateful to the late Dr. Martin Kalbe for the opportunity to visit the Max Planck Institute of Evolutionary Biology in Plön several times, to get firsthand laboratory experience with fish MHC analysis. I would also like to thank Dr. Irene Samonte-Padilla for her patience and guidance while teaching me the methods of MHC analysis of salmonids. While at Plön, Dr. Klaus Wächtler visited during my seminar on freshwater pearl mussels. We met again at Hamburg Airport when I went to deliver juvenile mussels to him. On both these occasions we had enjoyable and enlightening conversations about pearl mussels, and for this I would like to express my thanks.

At the Norwegian Institute for Nature Research I would like to thank Bjørn Mejdell Larsen for his help and feedback on this thesis. I would also like to thank Dr. Sten Ola Karlsson and Dr. Sebastian Wacker for their help with genetic analysis. Sebastian gave very helpful feedback on my third manuscript, and for this I would like to thank him.

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for proofreading my manuscripts many times. Last but not least, I would like to thank my husband Bjarne, for his love, support and understanding these many long years. I owe him my deepest gratitude for the summer holidays I was away harvesting mussels, for weekends he spent at the research station helping me to feed mussels or making sieves, for reading each and every manuscript so many times to help me make them perfect, for keeping our son fed and happy while I was away, and for his never-ending patience, encouragement and endurance.

List of Publications

Marwaha, J., Jensen, K.H., Jakobsen, P.J. and Geist, J. (2017). Duration of the parasitic phase determines subsequent performance in juvenile freshwater pearl mussels (*Margaritifera margaritifera*). Ecology and Evolution, 7 (5), pp 1375-1383. doi:10.1002/ece3.2740.

Marwaha, J., Aase, H., Geist, J., Stoeckle, B.C., Kuehn, R. and Jakobsen, P.J. (2019). Host (*Salmo trutta*) age influences resistance to infestation by freshwater pearl mussel (*Margaritifera margaritifera*) glochidia. Parasitology Research, 118 (5), pp 1519-1532. doi:10.1007/s00436-019-06300-2.

Marwaha, J., Jakobsen, P.J., Karlsson, S., and Wacker, S.W. (2019). Differential glochidial virulence and host bias of individual mothers observed in the freshwater pearl mussel (*Margaritifera margaritifera*) salmonid host-parasite system. (Manuscript)

Summary

The freshwater pearl mussel, *Margaritifera margaritifera*, is an endangered bivalve which has suffered a serious decline across its Holarctic distribution. It has a complex life cycle which involves an obligate parasitic stage on a suitable host. *M. margaritifera* populations are very host specific, and they are able to metamorphose only on the gills of Atlantic salmon (*Salmo salar*), sea trout (*Salmo trutta f. trutta*) or brown trout (*S. trutta f. fario*). Currently, the main concern is the lack of juvenile recruitment and survival in organically enriched river sediments. Consequently, several conservation programmes are rearing mussels in hatcheries, for eventual release back into their natural habitat when they are older and better able to survive.

Although *M. margaritifera* do not reproduce on their hosts, their survival is highly dependent on the presence of suitable hosts. The main purpose of this study was to improve our understanding of the host-parasite interactions and their influence on glochidial or juvenile mussel fitness, with the aim of providing information that could be used to refine future conservation strategies.

In the first experiment, the duration of the parasitic phase had a significant positive influence on post parasitic fitness of juvenile mussels in eight populations in Norway. Fitness was measured as size at excystment, post parasitic growth and survival. The strong positive relationship observed between the test variables clearly indicated that glochidial growth and development were dependent on individual host-parasite compatibility. In the same experiment, temperature was also observed to be an important factor governing excystment of juvenile mussels, with higher temperatures decreasing the duration of the parasitic phase. The variation in host suitability has been linked to environmental conditions, host age and/or size, genetic composition of the host and parasite, or a combination of these factors. Therefore, in the second experiment, the effect of host age on glochidial infestation was examined under common garden conditions. Hosts (0+ and 1+) were infested with glochidia from closely related mothers in order to remove the confounding effects of genotype-specific host interactions. A host age dependent immune response was observed, i.e.

the 0+ hosts displayed a resistant strategy, whereas the 1+ hosts displayed a tolerant one. In the second and third experiments, the virulent effects of glochidia on their hosts were examined, and measured as haematocrit values and host mortality respectively. Haematocrit values were significantly elevated in heavily infested hosts, which indicated respiratory distress (Chapter 3). In addition, glochidia were highly virulent on the less suitable host species, resulting in high mortalities of infested hosts (Chapter 4). Both these results display the parasitic nature of *M. margaritifera* in the host-parasite interaction. In the third experiment, the hypothesis that glochidia from a single mother could infest both salmon and trout hosts was examined. The results showed that glochidia from a population that uses salmon as its principal host were able to infest both species, but some mothers displayed a bias for either salmon or trout. These observations were probably a result of the higher genetic diversity observed in salmon-mussel populations. Individual and population level genetic diversity is associated with species fitness and an ability to adapt to a changing environment, which can help ensure long-term survival.

All the results of this study clearly indicate that the degree of host-parasite compatibility has an influence on glochidial and juvenile mussel fitness. Further studies should investigate factors that influence host-parasite interactions, for example the diversity of the genes of the host major histocompatibility complex (MHC). Based on the results, it is recommended that naive 1+ hosts are preferably used in captive breeding programmes, as this will maximise the production of juvenile mussels. The use of high quality hosts will also minimise the possible selection and genetic drift effects. Such effects lead to a deterioration in the evolutionary potential to adapt to a changing environment. Since glochidial development and successful metamorphosis into juvenile mussels is highly dependent on good host condition and survival, it is recommended that conservation efforts should focus on methods that can guarantee this.

An Ode to Margaritifera

Margaritifera margaritifera
So beautiful is thy name.
The beautiful pearl you create
Gives you thy unfortunate fame.

A very long life you have
Close to 200 years you can live.
An umbrella species you are
Filtered water to your surroundings you give.

You are born as a tiny glochidium
You will start your life as a parasite.
Floating along aimlessly
Waiting for a salmon or trout gill to bite.

Now begins the next stage of your journey
As you get encysted on the gills of your host.
You sextuple in size, in just eleven months
That's something of which you can boast.

A small white replica of your parents
You break free from your confining cocoon.
You bury yourself into the river bed
Usually in the beginning of June.

For five long years you stay hidden there
But soon you must take your place.
On the surface of the river bed
Life as a filter feeder, you must now embrace.

First we murdered your ancestors in thousands
For a pearl to bejewel our crown.
Then we destroyed and polluted your clean rivers
Your numbers just kept going down.

But now that you are an endangered species
To protect you is what we must do.
We have started nurseries for you and your young
Until the rivers we clean for you.

We want to restore you to your former glory
We want our children to see.
The fascinating life of the freshwater pearl mussel
In a river as it was meant to be.

Illustration by Elsa Beskow. Reprinted with permission from Floris Books.

1. General Introduction

"Each species is a wonder to behold, a long, brilliant history in itself to read, a champion emerged in our time after a long struggle of thousands or millions of years, best of the best, an expert specialist in the niche of the natural environment in which it lives," – E.O. Wilson in his book Half-Earth: Our Planet's Fight for Life (2016).

1.1 Freshwater bivalves – ecological importance

Freshwater mussels (Unionida) are large sedentary filter feeding mussels that are considered ecosystem engineers because of the important services they provide in freshwater ecosystems (Strayer et al., 1999; Bauer, 2001a; Vaughn & Hakenkamp, 2001; Bogan, 2008; Boeker et al., 2016; Lummer et al., 2016; Richter et al., 2016; Vaughn, 2018; Vaughn & Hoellien, 2018). Their ability to filter large quantities of water removes significant amounts of blue-green algae, diatoms, bacteria, fine particulate organic particles and silt from the water column, thereby improving the water quality in their surrounding habitat (Strayer et al., 1999; Vaughn & Hakenkamp, 2001; Bogan, 2008; Strayer, 2008; Vaughn et al., 2008; Lummer et al., 2016; Vaughn, 2018; Vaughn & Hoellien, 2018). In addition, their biodeposition and excretion of faeces increase the nutrient availability for other organisms in the water column (Vaughn & Hakenkamp, 2001; Vaughn et al., 2008; Vaughn, 2018; Vaughn & Hoellien, 2018). Freshwater bivalves, when found in dense aggregations, stabilize the sediment and their shells provide a habitat for epiphytic and epizoic organisms, and a refugia for benthic organisms (Ziuganov et al., 1994; Vaughn & Hakenkamp, 2001; Spooner & Vaughn, 2006; Vaughn et al., 2008; Vaughn, 2018; Vaughn & Hoellien, 2018). Bioturbation increases the water and oxygen concentration in the interstitial water, and also releases nutrients from the sediment into the water column (Vaughn & Hakenkamp, 2001; Spooner & Vaughn, 2006; Vaughn et al., 2008). Freshwater bivalves are thus an important part of freshwater ecosystems because they improve the habitat for all the organisms in their surroundings. They are especially important in habitats that are nutrient limited (Atkinson et al., 2013). Anthropogenic

disturbances have led to a significant global decline in freshwater bivalves over the last decades, with many species facing extinction (Bogan, 1993; Strayer et al., 1999; Lydeard et al., 2004; Dudgeon et al., 2006; Graf & Cummings, 2007; Bogan, 2008; Geist, 2010; Strayer & Dudgeon, 2010; Lopes-Lima et al., 2014; Vaughn, 2018). A decline in large dense aggregations of these bivalves will result in the loss of important "services", which could have a negative impact on functioning freshwater ecosystems (Howard & Cuffey, 2006; Vaughn, 2010; 2018).

1.2 The freshwater pearl mussel – *Margaritifera margaritifera*

Margaritifera margaritifera is an endangered freshwater bivalve which has already seen a serious decline across its Holarctic distribution (Araujo & Ramos, 2000; Machordom et al., 2003; Strayer et al., 2004; Geist, 2010; Boon et al., 2019). It is found from the Arctic to the temperate regions in western Russia and westwards through Europe to the north-eastern seaboard of North America, between 40°N and 70°N (Araujo & Ramos, 2000; Young et al., 2001; Machordom et al., 2003; Skinner et al., 2003; Strayer et al., 2004; Geist, 2010; Taeubert & Geist, 2017). It is usually found in fast flowing unpolluted oligotrophic rivers with a mixture of pebbles and rocks, intermixed with pockets of sand (Bauer, 1988; Ziuganov et al., 1994; Ziuganov et al., 2000; Skinner et al., 2003; Cosgrove et al., 2016). It has a semi-infaunal way of life, and is an example of an extremely long lived invertebrate, with life spans that exceed 100 years (Ziuganov et al., 2000; Bauer, 2001b). However, populations can vary in age according to their geographical location. It attains the longest life span in the cooler Scandinavian climate, where individuals can reach an age of up to 280 years (Ziuganov et al., 2000; Mutvei & Westermark, 2001). This decreases as one travels south; the southernmost populations have a typical life span of 35 years (Miguel et al., 2004).

The global decline in *M. margaritifera* populations has been attributed to anthropogenic activities that result in habitat degradation, alteration or fragmentation, changes in river/stream hydrology, geomorphology and physiochemical properties, and a decline in suitable host species (Cosgrove et al., 2000; Cosgrove & Hastie,

2001; Hastie & Cosgrove, 2001; Hastie et al., 2003; Geist et al., 2006; Moorkens et al., 2018). A major current concern is the lack of recruitment and survival of juvenile mussels, mainly due to eutrophication, siltation or acidification of rivers (Bauer, 1988; Hastie et al., 2000; Geist & Auerswald, 2007; Dolmen & Kleiven, 2008; Larsen, 2010; Magerøy, 2017; 2018; Magerøy & Larsen, 2019). The substrate requirements of juvenile mussels make them particularly sensitive to high levels of silt, suspended solids, biochemical oxygen demand, and organic pollution (Hastie et al., 2000; Geist & Auerswald, 2007; Dolmen & Kleiven, 2008; Cosgrove et al., 2016).

Listed on the IUCN Red List of Threatened Species, Annex II and V of the European Habitats and Species Directives (Directive 92/43/EEC), and Appendix III of the Bern Convention, *M. margaritifera* has become the focus of several national and international conservation actions (Jungbluth et al., 1985; Young et al., 2001; Machordom et al., 2003; Skinner et al., 2003; Larsen, 2005; Geist, 2010). Conservation efforts include restoration and protection of mussel habitats, release of artificially infested host fish and rearing of juvenile mussels followed by their release into the natural habitat (Ziuganov et al., 1994; Hastie et al., 2000; Preston et al., 2007; Bolland et al., 2010; Schmidt & Vandrè, 2010; Gum et al., 2011). Artificial rearing programmes are active in Austria, Belgium, the Czech Republic, Finland, France, Germany, Ireland, Luxembourg, Norway, Spain, and the UK. According to Strayer et al. (2019), artificial propagation of a larger number of freshwater pearl mussels has been considered a “major triumph for the conservation and management of these imperilled animals”.

Conservation strategies for the endangered *M. margaritifera* populations depend on a better understanding of habitat requirements for juvenile mussels, host requirements and glochidia-salmonid host interactions (Skinner et al., 2003; Geist & Auerswald, 2007; Geist & Kuehn, 2008; McIvor & Aldridge, 2008; Taeubert et al., 2010; Taeubert & Geist, 2017).

1.3 Life cycle of *M. margaritifera*

M. margaritifera have a life cycle that includes an obligate parasitic stage on a suitable host (Figure 1.1) (Meyers & Milleman, 1977; Young & Williams, 1984a; Ziuganov et al., 1994; Larsen, 2005; Geist, 2010; Taeubert et al., 2010; Taeubert & Geist 2017). They are usually dioecious, but females can switch to hermaphroditism when population densities are very low (Bauer, 1987). Males eject spermatozoa into the water column, and this is inhaled by females via their inhalant siphon. Fertilization occurs in the female brood chambers (Ziuganov et al., 1994). Larval glochidia develop while retained in the female brood chambers, and are then released into the water column when they are 60–80 μm in size (Ziuganov et al., 1994; Moorkens, 1999; Skinner et al., 2003; Wächtler et al., 2001). An adult female can produce up to 3–4 million glochidia per year (Young & Williams, 1984a; Wächtler et al., 2001), thus providing them with a high reproductive potential. Glochidial release is typically triggered by abrupt changes in the hydrological conditions of the river, causing a change in temperature or water quality parameters (Wellmann, 1943; Hastie & Young, 2003a). Once released, infective glochidia may remain viable for up to ten days (Jansen et al., 2001). During this time they passively attach to any object (including e.g. wood, plastic, or paper) (Kat, 1984; Dodd et al., 2005). In order to survive, however, glochidia must attach to the gills of a suitable host fish (Young & Williams, 1984a; Wächtler et al. 2001; Taeubert et al., 2010; Taeubert et al., 2013). After a parasitic period of 9–11 months, juvenile mussels (400–500 μm) excyst and spend the next five years buried in the river sediment, after which they rise and develop into adults (Smith, 1976; Bauer, 1987; 1994; Bauer & Vogel, 1987; Nezlin et al., 1994; Moorkens, 1999; Hastie & Young, 2003b; Geist, 2010). Adult mussels reach sexual maturation between the ages of 12–20 years (Young & Williams, 1984a; Bauer, 1987).

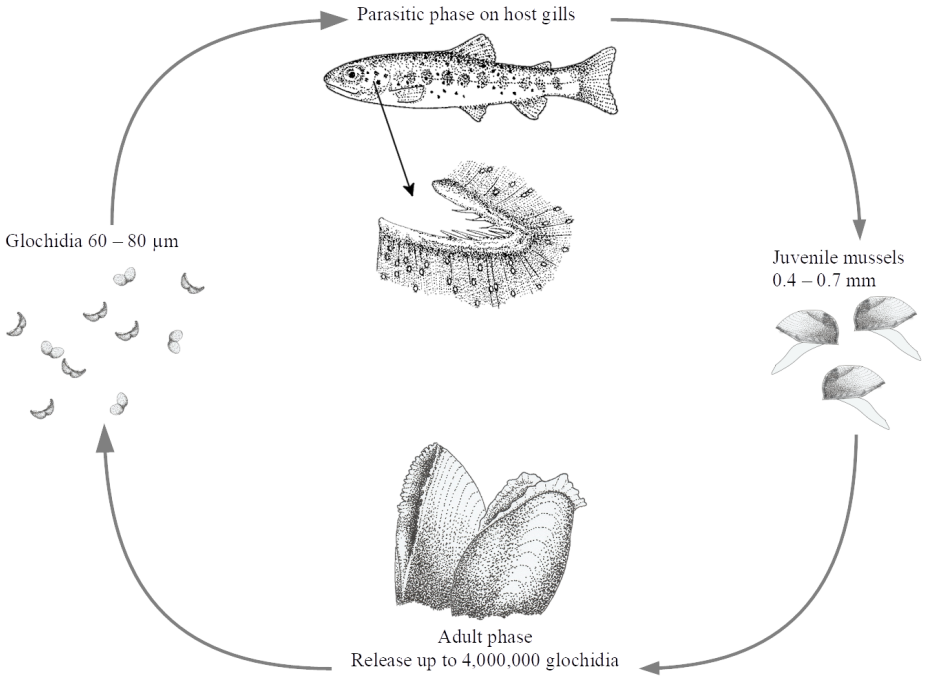

Figure 1.1: Life cycle of the freshwater pearl mussel *Margaritifera margaritifera*. Illustrations by Ragnhild Aakre Jakobsen.

1.4 The host of *M. margaritifera*

M. margaritifera is a specialist parasite that can only metamorphose on the gills of Atlantic salmon (*Salmo salar*), sea trout (*Salmo trutta f. trutta*) and brown trout (*Salmo trutta f. fario*) in its European distribution (Young & Williams, 1984b; Larsen, 2005; Geist et al., 2006), and brook trout (*Salvelinus fontinalis*) in North America (Smith, 1976; Taeubert & Geist, 2017). Brown trout has been observed to be the exclusive host for many central European populations, whereas Atlantic salmon is the exclusive one in some parts of northern Europe (Bauer, 1987; Geist et al., 2006; Ieshko et al., 2016). The salmonid host species preferred by populations can vary between different rivers, as well as between populations that occupy different parts of the same river (Larsen et al., 2000; Taeubert et al., 2010; Salonen et al., 2017; Taeubert & Geist, 2017). Moreover, some *M. margaritifera* exclusively infest either

Atlantic salmon (‘salmon-mussels’) or brown trout (‘trout-mussels’) even when both species are present, whereas others are able to use both species with varying degrees of suitability (Hastie & Young, 2001; Taeubert et al., 2010; Karlsson et al., 2014; Österling & Wengström, 2015; Ieshko et al., 2016; Salonen et al., 2017; Taeubert & Geist, 2017; Clements et al., 2018). In the latter instance, a population usually has one salmonid host species as the principal host, but they are able to infest a few specimens of the other, less suitable, salmonid host species (Clements et al., 2018). However, it is not known if glochidia from a single mother in such populations can infest both the principal and less suitable host, or some mothers exclusively infest salmon, and some trout.

Atlantic salmon is found along the east and west coast of the North Atlantic Ocean (Klemetsen et al., 2003). Although they are typically anadromous, examples of resident (landlocked) freshwater salmon populations are found in Norway, Sweden, Finland, Russia and North America (Klemetsen et al., 2003; Jonsson & Jonsson, 2011). Brown trout has a west to east distribution range that starts at the European Atlantic front and continues to the buttresses of the Himalayas (Baglinière, 1999; Lobón-Cerviá, 2017). The northernmost limit of its distribution range encompasses Iceland, Russia, Scandinavia, while the southernmost extends to the Atlas mountains (Morocco and Algeria) (Baglinière, 1999; Lobón-Cerviá, 2017). Brown trout populations are usually found in brooks, rivers and lakes, in mountainous as well as low lying areas, and also in estuaries and coastal seas (Jonsson & Jonsson, 2011). Resident brown trout are found only in freshwater habitats.

Atlantic salmon and brown trout are sibling species, and have similar life cycles. In autumn or winter, females deposit fertilized eggs in gravel nests that they have made in the river substrate (Gibson & Haedrich, 2006; Jonsson & Jonsson, 2011; 2017). Brown trout spawn earlier than Atlantic salmon, when both salmonids occur sympatrically. The eggs hatch into alevins the following spring, and spend the first month of their life in the river gravel (Jonsson & Jonsson, 2011). High water flow through the substrate provides the developing embryos, and later the alevins, with dissolved oxygen, and washes away metabolic waste (Jonsson & Jonsson, 2011;

2017). Once the yolk sacs are used up, the alevins (~20 mm) are ready for external feeding, and they emerge from the substrate as fry (0+ fish) (Hastie & Young, 2003c; Jonsson & Jonsson, 2011; 2017). The fry then develop into parr, and spend between 1–5 years in the river while feeding on epibenthic and drifting arthropods (Hastie & Young, 2003c; Jonsson & Jonsson, 2011). Parr transform into smolts when they are approximately 15 cm in size (Jonsson & Jonsson, 2011). Smolts develop a silvery belly and sides, and white pelvic fins, and migrate to the sea the following spring (Jonsson & Jonsson, 2011). In the next 1–4 years they grow into mature adults and return to their origin river to spawn. Atlantic salmon usually spawn every two years, whereas brown trout spawn every year (Jonsson & Jonsson, 2011). The salmonid life cycle stages and development can be influenced by water temperature, water flow and depth, bottom substrate, ice cover, migration barriers, nutrient richness and habitat (Jonsson & Jonsson, 2011). Detailed descriptions of the life cycles of Atlantic salmon and brown trout are provided by Jonsson and Jonsson (2011) and Lobón-Cerviá and Sanz (2017).

1.5 *M. margaritifera* in Norway

Historical records (written and oral) show that *M. margaritifera* has been present in Norwegian rivers and streams since the 17th century (Larsen & Magerøy, 2019). Today, populations are usually found along the coast and in lowland areas, extending up to 70°N (Økland & Økland, 1997). In the last hundred years there has been a significant decline, causing several populations to become extinct, especially in the southern and south-eastern parts of the country (Dolmen & Kleiven, 1999; Dolmen & Kleiven 2004; Larsen, 2010). The freshwater pearl mussel is nevertheless still widespread in Norway, and many large populations are for instance found in the counties of Møre og Romsdal, Trøndelag and Nordland (Figure 1.2) (Larsen & Magerøy, 2019). Trøndelag has about a quarter of all the pearl mussel streams in Norway (Larsen & Magerøy, 2019). As of March 2019, confirmed reports show the presence of *M. margaritifera* in 419 streams, but the degree of recruitment varies (Larsen & Magerøy, 2019). Larsen (2010) examined recruitment in 74 Norwegian

streams, and observed that 35% of the streams had good recruitment, 31% had weak or uncertain recruitment, and 34% had no recruitment. Assuming that these streams are representative of the degree of recruitment in general, only about two thirds of Norwegian pearl mussel populations have some degree of recruitment (Larsen, 2010). The greatest threat to the recruitment of juvenile mussels is eutrophication and siltation (Magerøy, 2017; 2018), as previously found for other parts of Europe (Geist & Auerswald, 2007; Geist, 2010). This results in decreased oxygen levels in the substrate (Magerøy, 2017; 2018). Moreover, acidification of rivers, especially prevalent in southern parts of Norway, has led to a decline of pearl mussel populations as well as host species (Hesthagen et al., 1999; Dolmen & Kleiven, 2008; Larsen, 2010).

Norway has an estimated quarter of the remaining *M. margaritifera* rivers, and about two thirds of the total number of individuals, in western Europe (Larsen, 2010). It therefore has a responsibility to protect and conserve the freshwater pearl mussel. *M. margaritifera* is listed as "vulnerable" on the Norwegian Red List of Species and has been designated as a "responsibility species" (Kålås et al., 2006; Larsen, 2010). Norway has its own action plan for the conservation of *M. margaritifera* populations which outlines the proposed measures for monitoring and improving habitats, public information and improving management routines, starting with the first plan published in 2006 (Larsen, 2005; Direktoratet for naturforvaltning 2006). Since then, the amount of knowledge on the freshwater pearl mussel and supportive measures that preserve and increase populations has grown, and a new action plan has therefore been published for the years 2019–2028. This action plan outlines the proposed measures for i) mapping and monitoring, ii) organisation, databases, information and guidance, iii) cultivation, iv) liming and v) cooperation among administrative agencies and the use of legislation. The main aim of the action plan is to ensure the long term survival of viable populations (Larsen, 2018; Direktoratet for naturforvaltning 2018).

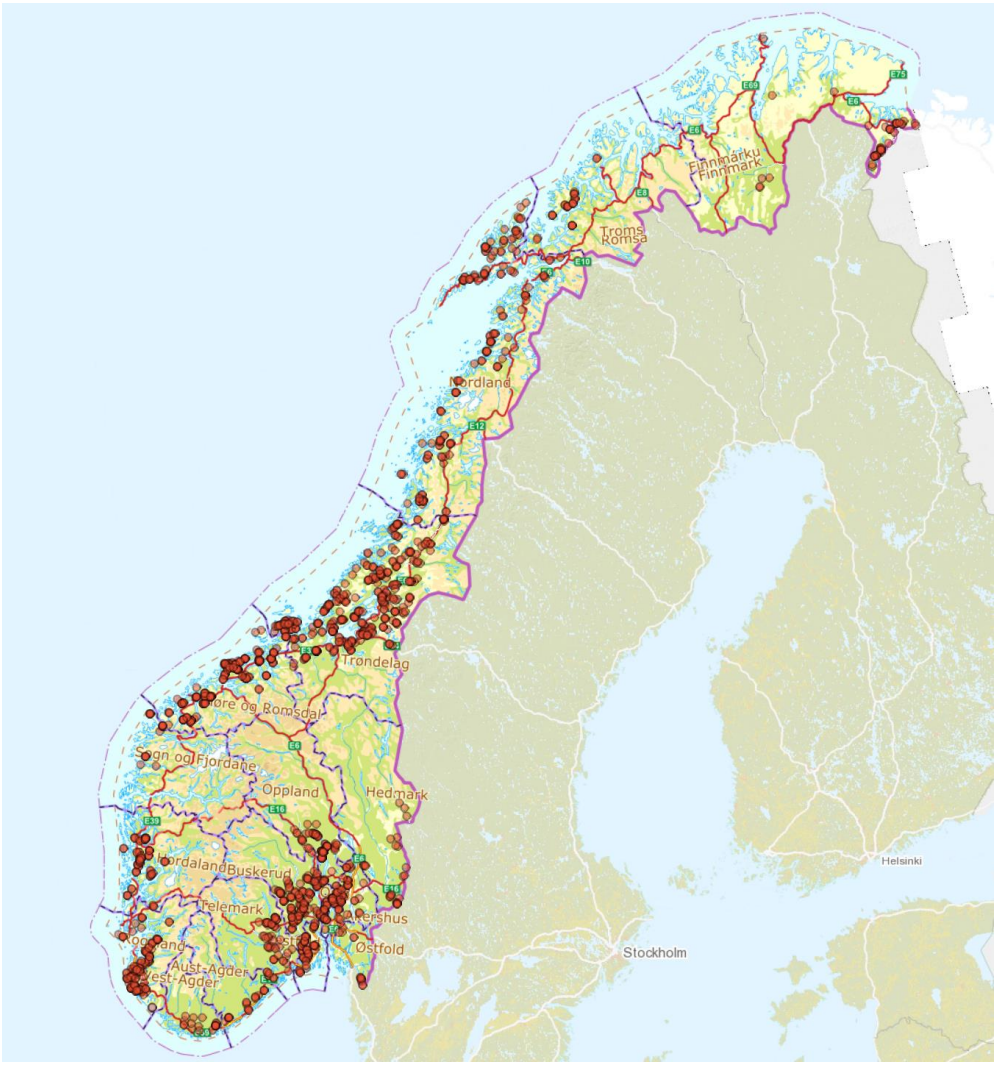

Figure 1.2: The distribution of *Margaritifera margaritifera* in Norway. The dots represent localities. Data obtained from https://www.artsdatabanken.no.

Field and experimental studies have shown that *M. margaritifera* populations in Norway are adapted to either Atlantic salmon (*S. salar*) or brown trout (*S. trutta*) (Larsen, 2005; Karlsson et al., 2014; Wacker et al., 2019a). In rivers where Atlantic salmon dominates over anadromous and resident brown trout, it is also usually the principal host for *M. margaritifera* populations (Larsen, 2005; Karlsson & Larsen, 2013). Sea trout is usually the principal host in rivers where they are the dominant

fish species (Larsen, 2005; Karlsson & Larsen, 2013). Resident brown trout is the only host in rivers where they naturally occur and are the only host species present (Larsen, 2005; Karlsson & Larsen, 2013). Moreover, some Norwegian *M. margaritifera* populations are observed to exclusively infest either salmon or trout, even when both species are present (Larsen et al., 2000; Larsen, 2002). Wacker et al. (2019) verified this in an artificial infestation experiment where salmon- and trout-mussels were exposed to both salmonid host species in the same infestation tank, and only infested their preferred host.

1.6 Host-parasite interactions

When glochidia attach to the gills of a suitable host, they are able to induce an immune response in the fish host that causes a cyst to form around each glochidium. Glochidia that are unable to induce this immune response do not become encysted, and are shed off (Nezlin et al., 1994). On unsuitable hosts, 'abnormal' cysts are formed, which leads to the sloughing off or death of the glochidia (Rogers-Lowery & Dimock, 2006). Encystment is believed to provide nutrition and mechanical protection to the developing glochidia, and is essential for metamorphosis into free living juveniles (Arey, 1932a, 1932b; Ziuganov et al., 1994; Wächtler et al., 2001; Denic et al., 2015).

Given the high host specificity of *M. margaritifera* populations, host suitability studies are performed to identify the most suitable hosts for a population. Such studies have shown that glochidial prevalence, abundance and size vary significantly among different salmonid host species, strains and even among individual fish of a suitable species/strain (Taeubert et al., 2010; Österling & Larsen, 2013; Salonen et al., 2017; Taeubert & Geist, 2017; Clements et al., 2018; Huber & Geist, 2019a; 2019b). Variation is also seen in the duration of the parasitic phase. For example, the juvenile mussel excystment period (the period from when the first mussel falls until the last one falls) can last anything between seven days (Bauer, 1979) up to 148 days (Taeubert et al., 2013). This extended excystment period is surprising, given the highly synchronous nature of glochidial release, which occurs within a time span of

1–2 days (Wellmann, 1943; Bauer, 1979; Young & Williams, 1984b; Hastie & Young, 2003c). Furthermore, Eybe et al. (2014) observed that juvenile mussels that excysted early were smaller and had a poor survival, compared to those that excysted late. The variation seen in the many aspects that relate to the parasitic phase is believed to be a result of host-parasite compatibility (Taeubert et al., 2010; Haag, 2012).

Host-parasite compatibility could be influenced by several factors, such as the genetic composition of the host and parasite, host factors (such as species, age, size, condition, infestation history, immune response, presence of other parasites), parasite factors (load, virulence), environmental conditions (such as temperature), or a combination of these (Bauer & Vogel, 1987; Combes, 2000; Taeubert, 2014) (Figure 1.3). The host immune response has been reported to be an important factor that influences glochidial metamorphosis. High mortalities are usually observed during the early stages of glochidial encystment, and only 5–10% of the attached glochidia metamorphose into free living juveniles (Hastie & Young, 2001). A large number of glochidia are usually lost 7 days post infestation as a result of the host mounting an immune response (Meyers et al., 1980; Bauer, 1987; Bauer & Vogel, 1987; O'Connell & Neves, 1999; Hastie & Young, 2003a). Another factor that affects compatibility could be previous glochidial infestation. Naive fish are believed to be better hosts for *M. margaritifera*, because previous glochidial infestations can result in acquired immunity (Karna & Millemann, 1978; Bauer, 1987; Bauer & Vogel, 1987; Bauer et al., 1991; Ziuganov et al., 1994; O'Connell & Neves, 1999; Rogers-Lowery et al., 2007; Thomas, 2011; Chowdhury et al., 2018). Previous studies have also examined the relationship between glochidial load (number of glochidia per fish) and host age or size (Karna & Millemann, 1978; Bauer, 1987; Bauer & Vogel, 1987; Bauer et al., 1991; Ziuganov et al., 1994; O'Connell & Neves, 1999; Rogers-Lowery et al., 2007; Thomas, 2011; Chowdhury et al., 2018). However, these studies have yielded contradictory results, and no clear relationship is established. As naturally infested wild fish were used in these studies, there could have been a bias in the results due to previous glochidial infestation.

Parasitic factors such as glochidial load and virulence can have an influence on the host-parasite interaction outcome. Glochidia spend between 9 to 11 months on their hosts, and their survival depends on their host's fitness and survival. Virulence is defined as reduction in host fitness (including host mortality) as a result of parasitic infestation (Bull, 1994; Read, 1994; Bieger & Ebert, 2009; Dybdahl & Storfer, 2003; Lambrechts et al., 2006). Virulent effects of glochidial infestation include an increase in host blood haematocrit values, spleen enlargement, respiratory stress and impaired swimming (Taeubert & Geist, 2013; Thomas et al., 2014; Filipsson et al., 2017). Low to moderate glochidial loads do not appear to have a significant detrimental effect on the hosts, however very high glochidial loads can lead to host mortality (Treasurer et al., 2006; Taeubert & Geist, 2013). Although the differences in host species or strain dependent susceptibility to glochidia are well documented (Larsen et al., 2000; Hastie & Young, 2001; Taeubert et al; 2010; Österling & Wengström, 2015; Salonen et al., 2017; Clements et al., 2018; Wacker et al., 2019a), host species dependent differences in glochidial virulence have not been examined. Moreover, glochidial virulence could also vary according to host age, i.e. older fish might tolerate infestation better than younger ones.

Most studies on host-parasite relationships involve short-lived parasites. Perhaps because of this, host-parasite interactions involving a long-lived parasite and the effect of these interactions on parasite fitness are not well understood. Conventionally, parasites are perceived as having a greater evolutionary potential and adaptive plasticity, resulting from them having larger population sizes, higher mutation rates, and shorter generation times compared to their hosts (Ebert, 1994; Kaltz & Shykoff, 1998; Gandon & Michalakis, 2002; Dybdahl & Storfer, 2003). In addition, a narrow host range and larger migration rates are generally believed to result in the parasite being locally adapted to its hosts (Dawkins & Krebs, 1979; Lajeunesse & Forbes, 2002; Dybdahl & Storfer, 2003; Kawecki & Ebert, 2004; Morgan, Gandon, & Buckling, 2005). *M. margaritifera*, however, is a long-lived specialist parasite which reaches maturity at the age of 12–15 years (Young & Williams, 1984b), whereas their salmonid hosts usually reach maturity at the age of

1–4 years (Jonsson & Jonsson, 2011). The reproductive lifespan of the host is thus about 30 times shorter than the parasite (Geist & Kuehn, 2008), and one would expect the salmonid hosts to have evolved strategies against the parasitic glochidia. However, the *M. margaritifera* salmonid host-parasite system has been stable for over 60 million years (Bauer, 1997).

In Lewis Carroll's *Through the Looking-Glass* (1871), the Red Queen tells Alice "Now, here, you see, it takes all the running you can do, to keep in the same place. If you want to get somewhere else, you must run at least twice as fast as that!" Van Valen (1973) observed a similarity between the Red Queen's advice to Alice and coevolution between biological enemies (Lively, 1996). Parasites and their hosts engage in a continuous 'arms race' to develop adaptations and counter-adaptations against one another (Mostowy & Engelstädter, 2011; Gokhale et al., 2013). Obligate parasites are under a strong selection pressure to infect the most common host genotypes, and the common host genotypes are pressured to resist them (King et al., 2011). If the parasite significantly reduces the fitness of the most common host genotypes, the latter's number decreases. This results in an increase in the numbers of the less common host genotypes, and the parasite must evolve in order to be able to infect the new common host (Lively, 1996; Gokhale et al., 2013; Rabajante et al., 2016). The parasite does not instantaneously adapt to the changes in the host populations, so there is typically a delay before they can infect the new common host genotype (Lively, 1996; Koskelly & Lively, 2009; Gokhale et al., 2013). This can result in "sustained oscillations in host and parasite gene frequencies and hence the maintenance of genetic variation" (Lively, 1996). The perpetual coevolution between the host and the parasite, in which neither wins the battle, is often referred to as the Red Queen hypothesis (Koskella & Lively, 2006; Rabajante et al., 2016; Aniza & Rabajente, 2018). The hypothesis suggests that coevolution will happen as a result of time-lagged negative frequency-dependent selection (Koskella & Lively, 2006; Rabajante et al., 2016; Anzia & Rabajante, 2018). In light of the Red Queen hypothesis, a question arises: How does the long lived parasite *M. margaritifera* keep up with its host when the host is 'running' at a much faster pace? Moreover, how does the high degree of specialisation of *M. margaritifera* (salmon-mussels and trout-

mussels) affect the ecology and future evolution of the parasite if the host composition is disturbed?

Taeubert and Geist (2017) suggested four possible evolutionary scenarios in the *M. margaritifera*-salmonid host interaction: i) there is no local adaptation and *M. margaritifera* can use any suitable salmonid host with similar success, ii) the shorter generation time and migratory behaviour of the salmonids will result in local adaptation of the host to the parasite, i.e. the sympatric hosts will have lower infestation rates, iii) the narrow host range of the parasite will result in *M. margaritifera* being locally adapted to their hosts, i.e. higher infestation rates on the sympatric hosts, or iv) a mixture of scenarios ii) and iii). Local adaptation is usually measured as the degree of parasite prevalence on the sympatric, compared to the allopatric, host (Dybdahl & Storfer, 2003). *M. margaritifera* populations are believed to be best adapted to their (historically) sympatric hosts, as suggested from infestation experiments (Taeubert et al., 2010; Salonen et al., 2017), as well as from similar genetic differentiation patterns among pearl mussels and their hosts (Geist & Kuehn, 2008). However, contradictory evidence has also been reported regionally (Österling & Larsen, 2013), and local adaptation in *M. margaritifera* populations has not yet been clearly demonstrated. In this host-parasite relationship, the parasite is expected to experience a stronger selection pressure on compatible host genotypes because its survival depends on host compatibility (Douda et al., 2017). In comparison, the hosts are expected to experience a weaker selection pressure for resistance host genotypes. This is because the parasite is distributed across a smaller area of the host's total distribution range, and they infest only the freshwater (young) stage of the host (Douda et al., 2017).

It is important to examine factors that influence these host-parasite interactions, because these may provide some answers about their role in the local adaptation of *M. margaritifera* populations, and contribute new information to help improve conservation efforts.

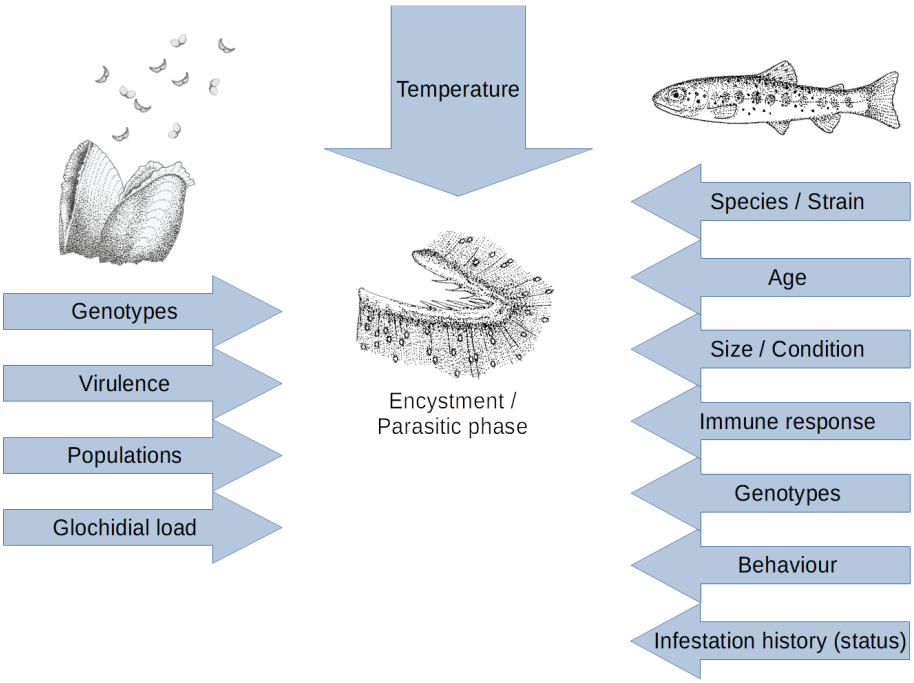

Figure 1.3: Host, parasite and environmental factors that can have an influence on the *M.margaritifera* salmonid host-parasite interaction. Illustrations by Ragnhild Aakre Jakobsen.

1.7 Objectives

Although *M. margaritifera* do not reproduce on their hosts, their life cycle is highly dependent on the availability of suitable salmonid host species. A very important step in their life cycle is glochidial encystment on the gills of a suitable host, without which glochidial metamorphosis into free living juveniles is impossible. High mortalities are observed during the early life stages of the *M. margaritifera* life cycle (Hastie & Young, 2001; Preston et al., 2007; Schmidt & Vandrè, 2010). Several authors have observed that i) 95–99% of the infective glochidia are not able to reach a suitable host and die, ii) only 5–10% of the encysted glochidia metamorphose into juvenile mussels, and iii) mortalities of excysted juvenile mussels can be as high as 95% (Young & Williams, 1984a, Hastie & Young, 2001, Preston et al., 2007,

Schmidt & Vandré 2010). The high mortalities in juvenile mussels are associated with their specific habitat requirements: a well aerated, clean and stable sediment (Hastie et al., 2000; Geist & Auerswald, 2007).

The complex life cycle and specific host and juvenile habitat requirements makes *M. margaritifera* particularly vulnerable to anthropogenic threats. Furthermore, their development and growth is dependent on water temperature (Hastie & Young, 2003a; Skinner et al., 2003; Österling et al., 2008; Taeubert et al., 2013), and temperature variations can disrupt reproduction (Hastie & Young, 2003a). Restoration of *M. margaritifera* populations rely on conservation efforts such as artificial propagation or restocking of infested fish hosts. In order to develop robust conservation methods, comprehensive studies on host-parasite interactions, as well as the underlying factors that influence the interaction outcome, are necessary. Thus, the overall objective of this study is to gain a better understanding of the host-parasite interactions between *M. margaritifera* and their salmonid hosts.

The following three experiments were performed:

1. The post parasitic stage is considered to be the most critical stage of the *M. margaritifera* life cycle (Hastie et al., 2000; Geist & Auerswald, 2007). Eybe et al. (2014) observed that juvenile mussels that had excysted at different times during the excystment period displayed differences in their size and survival. In the first experiment, the hypothesis that a longer duration of the parasitic phase increases fitness-related performance of juvenile mussels in their subsequent post parasitic phase was tested. Eight *M. margaritifera* populations were used to test this hypothesis. Moreover, being poikilothermic organisms, the developmental stages (spawning, brooding, glochidial development, growth, and release from the host fish) are dependent on the water temperature (Hastie & Young 2003a; Skinner et al., 2003; Österling et al., 2008; Taeubert et al., 2013). Therefore, the relationship between water temperature and juvenile mussel excystment rates was examined.

2. Several factors, such as environmental conditions, host age and/or size, genetic composition of the host and parasite, or a combination of these factors, can influence the outcome of host-parasite interactions. In the second experiment the hypothesis that salmonid hosts display an age-dependent response to glochidial infestation was examined. It was hypothesised that 1+ naive brown trout hosts tolerate glochidial infestation better than 0+ hosts. In addition, the relationship between glochidial load and haematocrit (% red blood cells in blood volume) values in the 1+ hosts was also examined in this experiment. It was hypothesised that heavy glochidial infestation would result in elevated haematocrit values, as a result of respiratory stress.

3. The negative effects of glochidial infestation on their hosts have been widely examined and include an increase in blood haematocrit values, spleen enlargement, respiratory stress, impaired swimming and impaired growth (Taeubert & Geist, 2013; Thomas et al., 2014; Filipsson et al., 2017; Chowdhury et al., 2019). In the third experiment, the hypothesis that glochidial infestation will result in higher glochidial virulence (measured as host mortality) in the less suitable salmonid host species was examined. In addition, the hypothesis that glochidia from a population with Atlantic salmon as its principal host are able to infest both the principal and less suitable salmonid hosts was examined.

2. Duration of the parasitic phase determines subsequent performance in juvenile freshwater pearl mussels (*Margaritifera margaritifera*)

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2.1 Abstract

Host-parasite systems have been useful in understanding coevolutionary patterns in sympatric species. Based on the exceptional interaction of the long-lived and highly host-specific freshwater pearl mussel (FPM; *Margaritifera margaritifera*) with its much shorter-lived host fish (*Salmo trutta* or *Salmo salar*), we tested the hypotheses that a longer duration of the parasitic phase increases fitness-related performance of mussels in their subsequent post parasitic phase, and that temperature is the main factor governing the duration of the parasitic phase. We collected juvenile mussels from naturally and artificially infested fish from eight rivers in Norway. Excysted juvenile mussels were maintained separately for each collection day, under similar temperature and food regimes, for up to 56 days. We recorded size at excystment, post excystment growth and survival as indicators of juvenile fitness in relation to the duration of the parasitic phase. We also recorded the daily average temperatures for the entire excystment period. We observed strong positive relationships between the length of the parasitic phase and the post parasitic growth rate, size at excystment and post parasitic survival. Temperature was identified as an important factor governing excystment, with higher temperatures decreasing the duration of the parasitic phase. Our results indicate that juvenile mussels with the longest parasitic phase have better resources (larger size and better growth rate) to start their benthic developmental phase, and therefore to survive their first winter. Consequently, the parasitic phase is crucial in determining subsequent survival. The temperature dependence of this

interaction suggests that climate change may affect the sensitive relationship between endangered FPMs and their fish hosts.

2.2 Introduction

Host-parasite systems have been extensively studied to understand coevolutionary processes. Hosts and parasites are in a continuous arms race against one another and are constantly developing adaptations and counter adaptations against each other. (Dawkins & Krebs, 1979). The survival of a parasite depends on successful infestation of, and establishment, on its host. The traditional view is that parasites have a greater evolutionary potential and adaptive plasticity resulting from larger population sizes, higher mutation rates, and shorter generation times compared to their hosts (Ebert, 1994; Kaltz & Shykoff, 1998; Gandon & Michalakis, 2002). In addition to these circumstances, a narrow host range and larger migration rates would most likely result in the parasite being locally adapted to its hosts (Dawkins & Krebs, 1979; Lajeunesse & Forbes, 2002; Kawecki & Ebert, 2004; Morgan et al., 2005). Most studies on host-parasite relationships involve short-lived parasites, but host-parasite interactions and their effect on parasite fitness are not well investigated in long-lived parasites. The unionoid freshwater pearl mussel (FPM; *Margaritifera margaritifera*) is one example of a long-lived specialist parasite, reaching ages of more than 200 years in its northern distribution range. With a generation time that is almost 30 times longer than its host (Geist & Kuehn, 2008), this host-parasite system allows for an interesting study of coevolutionary processes.

The FPM is an endangered bivalve that is listed in IUCN Red List of Threatened Species, Annex II and V of the European Habitats and Species Directives (Directive 92/43/EEC) and Appendix III of the Bern Convention (Machordom et al., 2003; Skinner et al., 2003; Larsen, 2005; Geist, 2010). A serious decline of FPM across its geographical range has attracted much concern from national and international conservation organizations (Araujo & Ramos, 2000; Machordom et al., 2003; Strayer et al., 2004; Geist, 2010). Conservation efforts for the species include habitat protection and restoration, release of artificially infested host fish and rearing of

juvenile mussels followed by their release into the natural habitat (Ziuganov et al., 1994; Hastie & Young, 2003c; Preston et al., 2007; Bolland et al., 2010; Schmidt & Vandrè, 2010; Gum et al., 2011). Rearing programmes for the FPM have been put in place in Austria, Belgium, the Czech Republic, Finland, France, Germany, Ireland, Luxembourg, Norway, Spain and the UK. Current research is focused on understanding the bottlenecks in the life cycle, especially identifying host requirements (Skinner et al., 2003; Geist & Auerswald, 2007; Geist & Kuehn, 2008; McIvor & Aldridge, 2008; Taeubert et al., 2010; Taeubert & Geist, 2017). This knowledge could be useful in improving the understanding of coevolutionary host-parasite interactions as well as in developing improved culturing techniques that can aid conservation.

The complex life cycle of FPM comprises a short-lived drifting stage (infective glochidia), followed by an obligate parasitic stage on salmonids and a benthic stage during which juvenile mussels remain buried in the river sediment for around 5 years (Smith, 1976; Bauer, 1987; 1994; Nezlin et al., 1994; Ziuganov et al., 1994; Moorkens, 1999; Hastie & Young, 2003c; Geist, 2010). Although the general life cycle and glochidial larval stages have been described in detail, there are several aspects of parasite-host compatibility, including the influence of the host on the fitness and success of the parasitic (glochidial) and post parasitic (juvenile mussel) stages of the life cycle, which are not well understood (Taeubert & Geist, 2017).

Glochidia, 60–80 µm in size (Moorkens, 1999; Wächtler et al., 2001; Skinner et al., 2003), are released by gravid mothers and have to attach to the gills of a suitable fish host, where they become encysted and metamorphose (Arey, 1921; 1932a; b; Kat, 1984; Young & Williams, 1984b; Nezlin et al., 1994; Araujoet al., 2002; Dodd et al., 2005; Larsen, 2005; Geist, 2010; Taeubert et al., 2010; Taeubert et al., 2013). This release of glochidia has been reported to be a highly synchronous event with all gravid specimens from each river population releasing their glochidia within a time span of only 1–2 days (Wellmann, 1943; Bauer, 1979; Young & Williams, 1984b; Hastie & Young, 2003c). The release is typically triggered by abrupt changes in hydrological conditions of the river, causing a change in temperature or water quality

parameters (Wellmann, 1943; Hastie & Young, 2003c). FPM development and growth is generally dependent on water temperature (Hastie & Young, 2003c; Skinner et al., 2003; Österling et al., 2008; Taeubert et al., 2013) and temperature variation can delay reproduction within rivers by several weeks during cold years (Hastie & Young, 2003c). However, Hastie and Young (2003c) observed several rivers over several years and found glochidial release to be a synchronous event within the river every time. It is, therefore, expected that in rivers located in areas with similar temperature regimes, glochidial release occurs around the same time. Furthermore, once released the glochidia may remain viable for up to 6 days (Ziuganov et al., 1994; Jansen et al., 2001). However Young and Williams (1984b) observed that the glochidia became lifeless 24 hours post-release and in natural conditions glochidia only remain in suspension for a short period of time during which they have to infest their host.

In European FPM, glochidia can successfully metamorphose only on the gills of Atlantic salmon (*Salmo salar*), sea trout (*S. trutta f. trutta*) and brown trout (*S. trutta f. fario*) (Young & Williams, 1984b; Larsen, 2005; Geist, 2010; Taeubert et al., 2010: Taeubert et al., 2013; Ieshko et al., 2016). In addition it has been reported that FPM populations exclusively infest either Atlantic salmon or brown trout even if both species are present in the same rivers (Larsen et al., 2000; Karlsson et al., 2014; Ieshko et al., 2016). The length of the parasitic glochidial developmental phase is highly variable (Ziuganov et al., 1994). In FPM and other species of freshwater mussels, the duration of the host-dependent phase is expected to be related to either the temperature at which they develop, compatibility with the host, or both (Lefevre & Curtis, 1912; Ziuganov et al., 1994; Taeubert et al., 2010; Taeubert et al., 2013; Taeubert et al., 2014). Two glochidial developmental strategies have been described; one with a developmental period of 20–60 days (Bauer, 1979; Young & Williams, 1984b; Ziuganov et al., 1994) and one with a developmental period of 7–9 months (Bauer, 1979; Ziuganov et al., 1994). Both these developmental strategies have been observed within the same mussel population (Ziuganov et al., 1994). In Norway, the long developmental strategy is observed (Larsen, 2005). During the parasitic phase, glochidia grow 6–10 fold their original length (Moorkens, 1999; Taeubert et al.,

2013) and once they have reached a size larger than 240 µm, all organs of the adult mussel that are required for a benthic existence are present (Ziuganov et al., 1994). Juvenile mussels excyst at sizes between 280–500 µm (Bauer, 1994; Ziuganov et al., 1994; Hastie & Young, 2003c; Eybe et al., 2014; Marwaha, 2012, personal observation).

The length of the excystment period (which starts with the first and ends with the last juvenile mussel dropping off its host) is highly variable (Ziuganov et al., 1994; Taeubert et al., 2013; Eybe et al., 2014) and periods lasting from seven days (Bauer, 1979) up to 148 days (Taeubert et al., 2013) have been reported. We have observed excystment periods from 40 days up to 60 days for Norwegian FPM. The extended excystment period in juvenile mussels is surprising when considering the highly synchronous nature of glochidial release and the short life span of the released glochidia. It would be reasonable to assume that for one FPM population, hosts are infested within a very small time window. We might therefore have expected to see more synchronous excystment as well. Eybe et al. (2014) observed that larger mussels excyst at the end of the excystment period. In addition they also observed that the early excysters had a poor survival, but it remains unclear if this observation from one specific pearl mussel population can be generalized. In order to investigate whether this was a general trend across multiple populations, we used eight Norwegian FPM populations in our experiment. Additionally, we also wanted to observe whether there were any other fitness benefits associated with prolonged excystment.

The objective of this study was to investigate whether the timing of excystment (i.e. the amount of time elapsed since the first mussel excysted) had an effect on the survival and post excystment performance of juvenile pearl mussels from eight Norwegian FPM populations. In particular, we hypothesized that there is a positive correlation between the duration of the FPM parasitic phase on its host with its size and growth during the parasitic phase, but also with beneficial effects on subsequent survival and growth in the post parasitic phase. In addition, we hypothesized that temperature has a strong positive effect on excystment rates. By collecting results

from several FPM populations, we would be able to verify whether our hypothesis would hold true as a general trend observed in the FPM life cycle.

2.3 Materials and methods

In order to test our hypotheses, we used both naturally and artificially infested fish (*S. trutta f. fario* and *S. salar*). Naturally infested fish were collected from seven rivers (Table 1) in southern Norway by electro-fishing. The artificial infestations were performed in the river Haukåsvassdraget, where 30 gravid mussels and 100 young of the year farmed trout were kept in a holding tank and natural infestation was allowed to take place. In this case, all glochidial release was synchronous occurring within 2 days. All infested fish, natural or artificial, were transported to the mussel breeding station at Austevoll, Norway, and maintained there until we finished harvesting the juvenile mussels.

Water from the lake Kvernavatnet (Austevoll) was used for maintaining fish and juvenile mussels during the experiments. It has a pH of 6.6 and alkalinity of 0.108 mmol/l. Concentrations of aluminium, iron, calcium, magnesium and nitrate were as follows: Al – 180 µg/l; Fe – 200 µg/l, Ca – 4.2 mg/l, Mg – 1.8 mg/l, Na – 12 mg/l and Nitrate–N – 0.15 mg/l. The water was UV-treated and filtered through a 30 µm mesh before use. Since the water came from the lake, water temperature of the fish holding system followed the natural temperature variation of the lake and was between 5.7 and 17 ºC.

Infested fish were transferred and maintained in juvenile mussel collecting chambers until the end of the excystment period, following the methodology originally described by Hruška (1999). All infested fish from a single FPM population were kept in one juvenile mussel collecting chamber. The 200 µm collection sieves were inspected daily to check for the presence of excysted juvenile mussels (Figure 2.1). Once the excystment of mussels began, the collection sieves were examined every alternate day for the collection of juvenile mussels.

Mussel river population	Host fish	Number of fish	Type of infestation	Total mussels harvested
Haukåsvassdraget	*Salmo trutta f. fario*	55	Artificial	353
Hopselva	*Salmo trutta f. fario*	25	Natural	323
Lerangsbekken	*Salmo trutta f. fario*	10	Natural	241
Ereviksbekken	*Salmo trutta f. fario*	31	Natural	237
Steinslandselva	*Salmo salar*	49	Natural	376
Oselva	*Salmo salar*	30	Natural	630
Fossa	*Salmo trutta f. fario*	22	Natural	230
Åreidselva	*Salmo trutta f. fario*	24	Natural	490
			Total	**2880**

Table 1: The rivers of origin for each freshwater pearl mussel population, host fish species and number, type of infestation, and the total number of mussels harvested per river population.

Excysted mussels were collected and cleaned thoroughly, i.e. only living mussels devoid of all debris (such as fish faeces, teeth, scales, and small insects) were put into plastic boxes (175 × 116 × 97 mm) (Hruška, 1999). All the mussels from one population from a single collection day were kept separately in boxes (Figure 2.1). As the number of excysting mussels varied between each collection day (from a minimum of 2 to a maximum of 119), we decided to have an upper limit of 50 mussels per box. This resulted in boxes with different densities of mussels. Although Eybe et al. (2013) observed that mussel density can have an effect on performance, we did not observe such an effect in our experiment (see Results section). It needs to

be noted that Eybe et al. (2013) used much higher densities (200, 300 and 400 mussels per 500 ml box) compared to ours. All boxes were kept in a temperature-controlled room at a temperature of 17.0 ± 0.54 ºC (Figure 2.1). The juvenile mussels were fed every second day with a food mixture described by Eybe et al. (2013). In 10 litres of water we added 1 ml of calcium solution (2.7 mg/l), 250 µl of Shellfish® diet 1800 (Reed Mariculture Inc., Campbell, California, USA) and 2 ml of a stock solution containing 50 ml of tap water, 0.35 g spirulina (*Arthrospira platensis*) (Bio-life, Norway), 1 ml Nanno 3600 (Reed Mariculture Inc., Campbell, California, USA) and 10 crushed chironomid larvae (Eybe & Thielen, 2010; Scheder et al., 2014; Lange personal communication 2012). Feeding involved a water change in the box, i.e. removal of old food water, rinsing the boxes with clean water before adding 700 ml of food mixture and 100 ml of detritus. The detritus was obtained from a swamp around a small brook, near the breeding station. It was filtered through a 30 µm sieve and oxygenated for 3 days prior to use.

To investigate whether there was a post excystment fitness effect for juvenile mussels that excysted late, we measured the size at excystment, and post excystment growth rate and survival. For each FPM population, the total number of mussels that excysted on each collection day were counted and measured to the nearest 0.1 mm. The length of each juvenile mussel (defined as the maximum length of the shell at its greatest extension) was measured using a 10× calibrated ocular micrometer in a dissecting microscope. All juvenile mussels were measured on the day of excystment. To compare the growth rates of early and late excysters, juvenile mussels were measured between two time points (using the excystment time point as reference) and average growth rate per day was calculated as the increase in length (µm/day) using the absolute growth rate formula from Hopkins (1992). For assessing survival, we recorded the proportion of surviving juveniles in a given box, from the day of excystment until a given day post excystment. Because mortality is very low after the first week of excystment, we only recorded this endpoint between 22 and 33 days post excystment. Finally, temperature at excystment was recorded to test for links between temperature and number of excysting mussels.

All statistical analyses were computed using the statistical package R version 3.3.2 (R Development Core Team, 2016). To check whether there was a relationship between growth rate and duration of the parasitic phase (i.e. time on gills which was measured as the amount of time passed after the first mussel excysted in a given river), we first established a model with growth rate as a response variable and with the predictors size at excystment and density of mussels. We then used the residuals of this model tested against time on gills. We did this to control for the effect of size and density of mussels. For both models, we used a linear mixed effect modelling (LME) with the river from which each mussel population originated as a random effect factor. To check whether there was a relationship between mean size at excystment and duration of the parasitic phase (time on gills), we used the same type of model (LME) where river was set as a random effect factor. A generalized linear mixed effects model (GLMM) with quasibinomial error term was used to investigate the relationship between the survival during the non-parasitic phase (post excystment) and the duration of the parasitic phase. As in the previous models, the river from which the mussels originated was set as a random effect factor The response variable in this model was the proportion of survivors in a given mussel box until a given post parasitic age ranging from 22–33 days depending on when the boxes were checked for survival. Since survival was not checked at a fixed post parasitic age, we analysed the data with post parasitic age as a covariate in the model to control for eventual effects of this variable. A GLMM approach with river as a random effect factor was also used to test the relationship between the number of mussels excysting and the temperature. In this model, Poisson was set as an error term as the response variable represents count data. All the above statistical methods are described in Zuur et al. (2009).

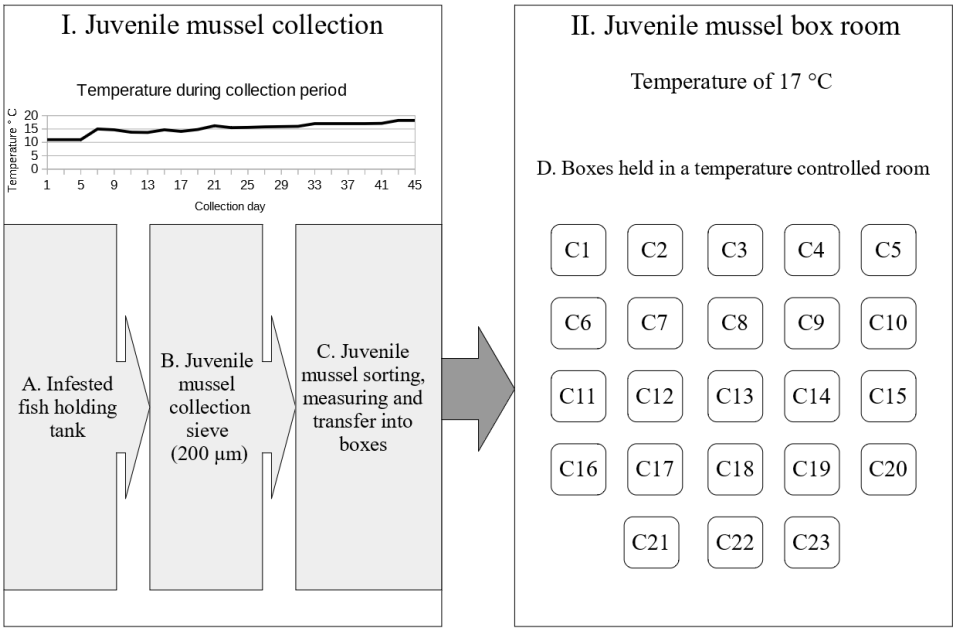

Figure 2.1: Schematic overview of methods used for each freshwater pearl mussel (FPM) population for a single collection day applied for a total of 24 collection days. Box I: Procedure for juvenile mussel collection. (A) Fish holding tank with infested fish (1 FPM population/tank). (B) Mussel collection sieve (200 µm) from which excysted mussels (end of parasitic phase) were collected every alternate day. (C) Excysted mussels were cleaned, counted and measured (size) and put into boxes (C1 – C23) (50 mussels/box). Temperature panel shows the temperature for the different collection days. Box II: (D) Temperature-controlled mussel box room with boxes from the collection days (C1 – C23). Temperature kept constant at 17.00 ± 0.54 ºC.

2.4 Results

The duration of the parasitic phase (time on gills) had a positive effect on growth rate (LME: $F_{1,128} = 5.54$, p-value = 0.02, Figure 2.2). However, the variability over time on gills was large and there were some individual mussels that dropped off early and at a small size which had higher growth rates compared to those that excysted later and at larger sizes. The model revealed a relatively low effect of individual rivers,

where the estimated between river standard deviation was 0.82 and the estimated within river standard deviation was 2.07.

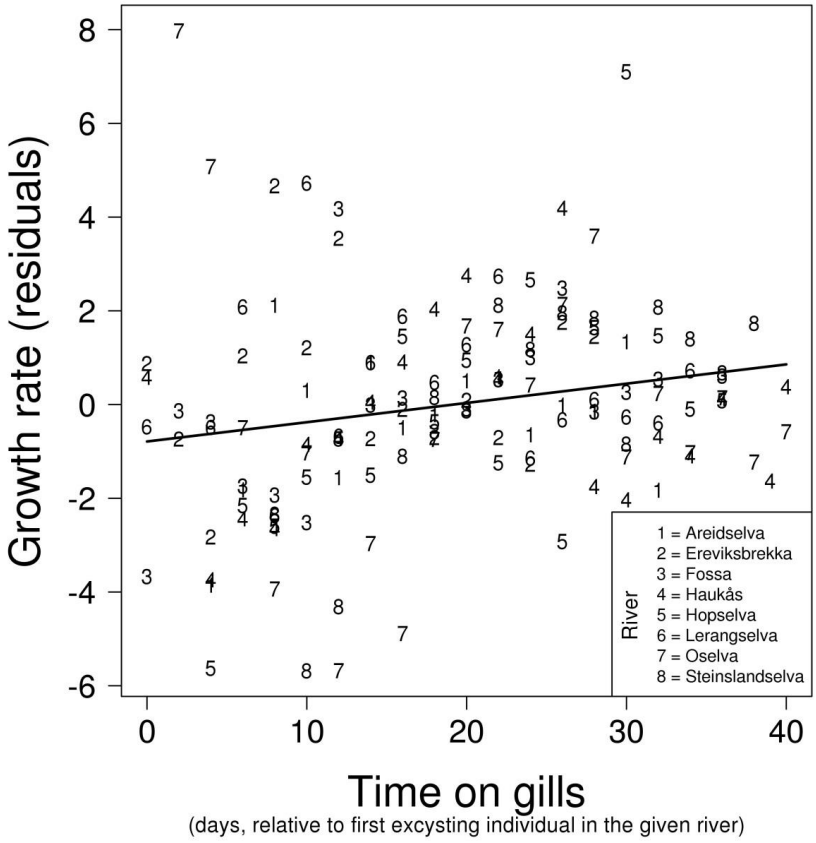

Figure 2.2: Relationship between time of excystment and residual growth rate (µm/day). The residuals are from a model with size and mussel density as predictors. The line represents model predictions and different symbols indicate different rivers.

In addition we also observed a positive relationship between the duration of the mussel parasitic phase and their mean size at excystment (LME: $F_{1,137} = 379.30$, p-value < 0.01, Figure 2.3). The mussels that dropped off at the end of the excystment period (42 days after the first one excysted) were larger than the first excysters by a factor of 1.49. The estimated between river standard deviation was 0.02, while the estimated within river standard deviation was 0.03.

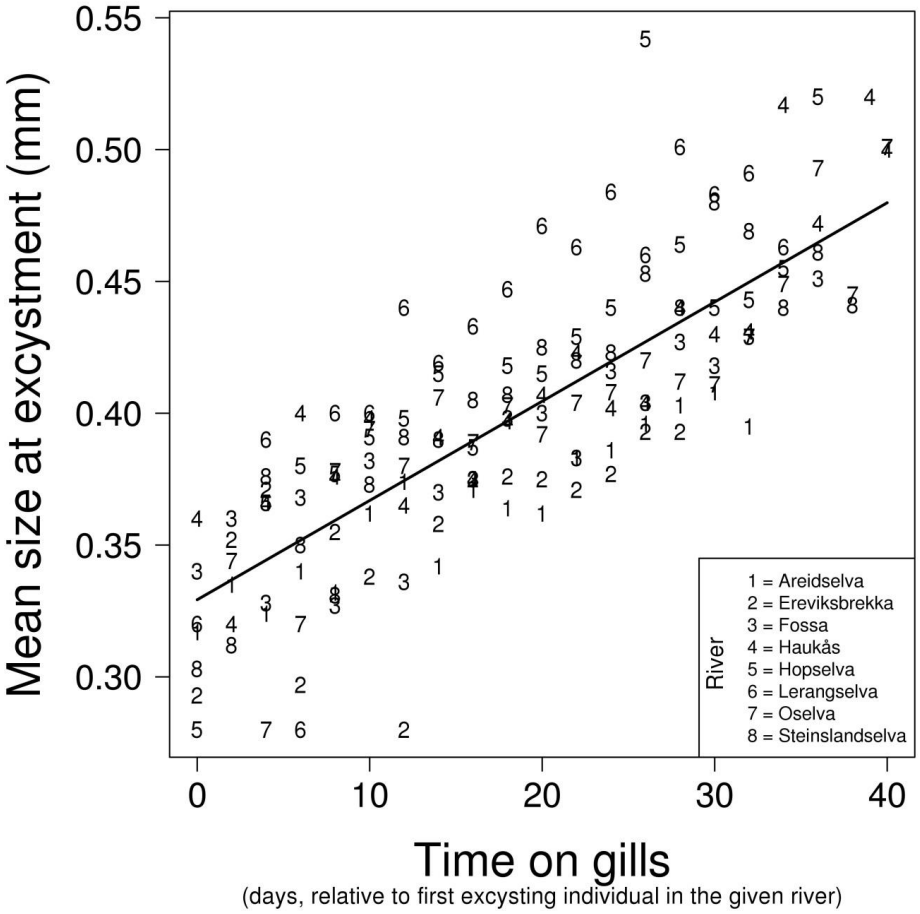

Figure 2.3: Relationship between the time that mussels spent on the host fish (day 0 refers to the day when excystment started in a given river) and their mean size at excystment.

The generalized linear mixed effect model used to examine the post parasitic phase survival depending on the duration of the parasitic phase showed a positive relationship between the duration of the parasitic phase (time on gills) and survival (GLMM: t-value = 4.32, d.f. = 100, p-value = 0.02, Figure 2.4). The estimated between river standard deviation was 0.41, while the estimated within river standard deviation was 0.59.

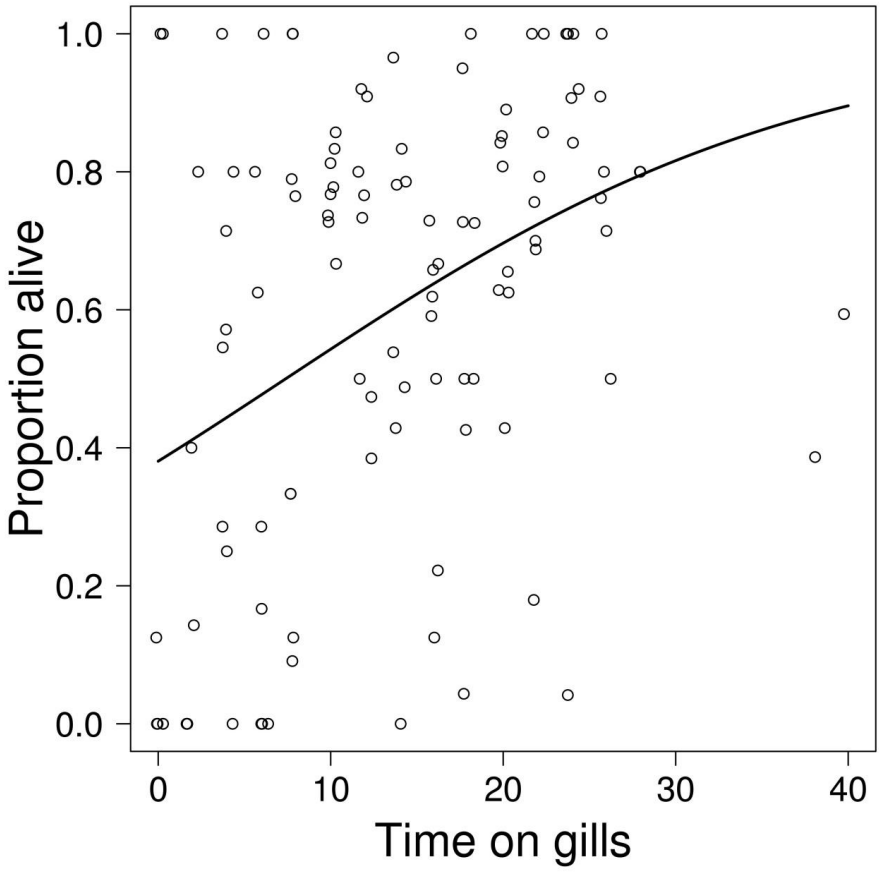

Figure 2.4: The proportion of survivors depending on the duration of the parasitic phase (time on gills).

There was a positive relationship between temperature and the number of mussels that excysted (GLMM: d.f. = 152, t-value = 6.05, p-value < 0.01, Figure 2.5) where the predicted number of excysted individuals at 11 and 18 ºC were 5.63 and 35.65 individuals, respectively. The estimated between river standard deviation was 0.33, while the estimated within river standard deviation was 3.43.

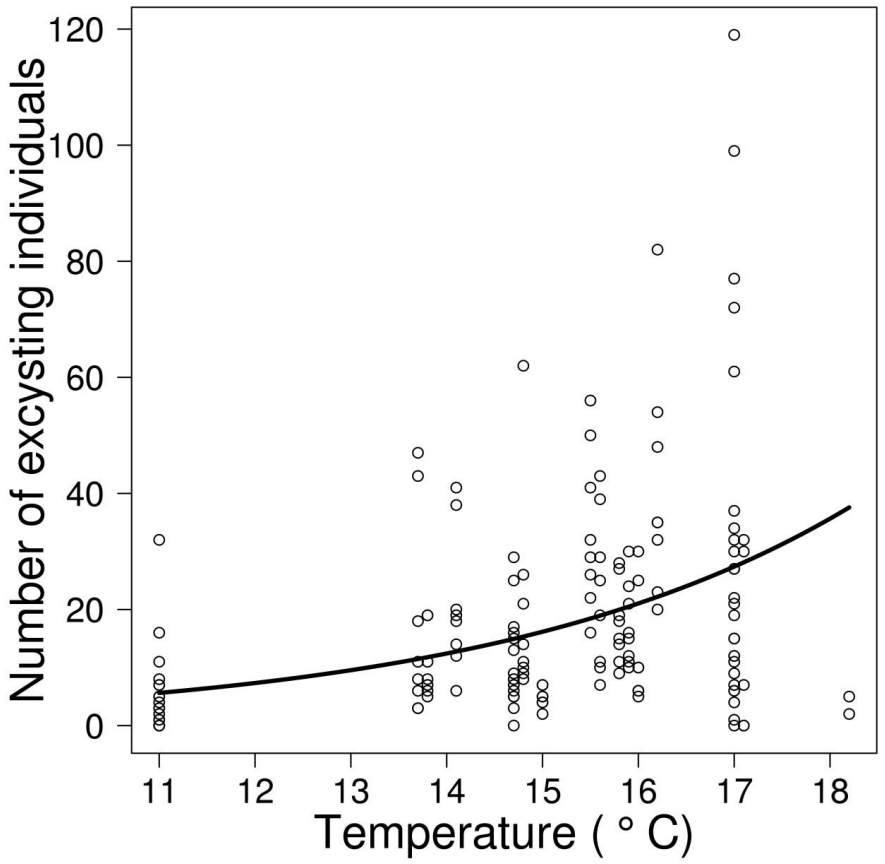

Figure 2.5: Number of excysting individuals depending on temperature.

2.5 Discussion

The results of this study suggest that the duration of the parasitic phase of FPM larvae on their fish hosts has positive effects on their subsequent size and growth rates. In addition juvenile mussels with a longer parasitic phase had higher survival rates. Moreover, and in line with previous studies (Taeubert et al., 2013), temperature was identified as an important driver governing the numbers of dropped-off juveniles. All the eight FPM populations that were investigated consistently showed these results.

In the case of the naturally infested fish, results may be confounded due to the asynchronous release of glochidia. However, this appears highly unlikely based on evidence from the literature and our observations of a highly synchronous release over several years for the populations under study (data not shown). We have also had parts of these FPM populations at the rearing facility in subsequent years and have observed that all glochidial releases occurred synchronously, within a period of 1–2 days. In addition several authors have also observed a similar release of glochidia (Wellmann, 1943; Bauer, 1979; Young & Williams, 1984b). Hastie and Young (2003c) also observed this behaviour over several years. Furthermore, all the FPM populations used in our experiment were from rivers in southern parts of Norway which have similar geographical, hydrological and temperature conditions.

Mussels that excysted later during the excystment period had clearly benefited in terms of size, post excystment growth and survival. Late excysters will most probably have better resources to start their benthic existence and hence have better survival (Österling & Larsen, 2013; Eybe et al., 2014). This would be particularly important during the first winter, especially in Norway and other areas with colder climatic conditions where winter temperatures are lower compared to central or southern Europe. Our results are in line with the practical observation that juvenile mussel survival during the first winter depends on the mussels attaining a critical shell length of 1 mm in order to survive it (Gum et al., 2011; Lange & Selheim, 2011).

The difference in fitness between the early and late excysters could be due to a variable developmental speed of the glochidia which in turn could be related to parasite-host compatibility. In a FPM-host suitability experiment, Taeubert et al. (2010) observed that the most suitable fish strain had higher infestation rates as well as highest glochidial growth rates. They also observed that glochidial sizes were highly different among individuals of the same host species/strain. They suggested that this was due to the differences in compatibility between the parasite and host. Parasite-host compatibility will influence the successful encystment of the glochidia, which is essential for a successful parasitic phase (Haag, 2012; Taeubert & Geist, 2017). When glochidia attach to the gills of the specific host, they elicit an immune

response and are then encysted by the fish host. However, those that cannot elicit an immune response from the fish host are not encysted and are shed off (Nezlin et al., 1994). On attaching to an unsuitable host an 'abnormal' cyst forms which leads to sloughing off or death of the glochidia (Rogers-Lowery & Dimock, 2006). The cyst is essential for the parasitic phase (Haag, 2012) because it is thought to provide nutrition and mechanical protection to the glochidia (Arey, 1932b; c; Ziuganov et al., 1994; Wächtler et al., 2001). Thus it is likely that the degree of compatibility with the host fish will influence how successfully the host builds the 'house' cyst around the glochidia, which in turn affects the establishment and degree of nutrition available to the developing glochidia. We believe that this parasite-host compatibility could be related to the major histocompatibility complex (MHC) variability of the fish hosts. It has been shown that MHC variability influences growth of parasites (Kurtz et al., 2004). Furthermore, we have observed (Marwaha et al., 2014 unpublished data) that juvenile mussels were larger on MHC heterozygous fish compared to MHC homozygous fish. Thus it is very likely that the success of glochidial encystment, and therefore growth and development, depends on the MHC variability of the fish hosts.

Other factors could also influence the availability of nutrition to the developing glochidia (Taeubert et al., 2013). For example, the position of the cyst on the gills of the host fish might be important. Glochidia encysted on the gill rakers could have different nutrition available compared to those on the gill filaments. In turn, this could influence developmental speed (Taeubert et al., 2013).

The lower survival we observed in juvenile mussels with a short parasitic phase is most probably related to premature excystment (Eybe et al., 2014). Eybe et al. (2014) proposed that mussels, while still encysted, continue to grow during the excystment period by continuously taking up nutrients from their host. Premature excystment could result in small, poorly developed mussels that are unable to survive the first month in their benthic habitat.

In line with other reports (Taeubert et al., 2013), we also observed that temperature was an important environmental cue for excystment. There is likely an optimal time

for excystment of mussels in relation to water temperature, i.e. at the ideal temperature the maximum numbers of mussels will excyst. Buddensiek (1995) observed that juvenile mussel growth was restricted to the warmer months of the year and they stopped growing in the cold winter months, a pattern that results in tree-ring like growth structures in the mussel shells (Geist et al., 2005). Therefore, it would be beneficial for a mussel to excyst at a temperature at which the juvenile mussels can start their benthic stage under ideal conditions and benefit from maximum growth before the winter period.

With the development and growth of FPM being dependent on water temperature (Hastie & Young, 2003c; Skinner et al., 2003; Österling et al., 2008; Taeubert et al., 2013), variation in temperature can influence glochidial metamorphosis (Hruška, 1992; McIvor & Aldridge, 2008), growth (Larsen, 2005), duration of the parasitic phase and release of glochidia from their cysts (Lefevre & Curtis, 1912; Hruška, 1992; Ziuganov et al., 1994; Larsen, 2005; McIvor & Aldridge, 2008 Eybe et al., 2014). Reproduction stages of FPM are thought to depend on either a critical minimum water temperature or a summation effect ('minimum number of cumulative day-degrees') or both these factors (Jungbluth & Lehmann, 1976; Hastie & Young, 2003c). Thus any change in the natural temperature regime (e.g. due to climate change) can affect the sensitive relationship between parasite and host which is particularly crucial in the context of conservation of the endangered FPM. Although our data suggest that temperature appears to be the most important factor which influences the glochidial development and timing of the start of excystment, it does not explain why the post excystment growth, under equal temperature conditions, is higher in those mussels that excyst late. This observation can only be explained by other factors such as the previously discussed parasite-host compatibility.

Some mussel populations have prolonged excystment periods. This could be advantageous, as it allows for the dispersal of juvenile mussels over a larger river area through host migration (Watters & O'Dee, 1999; Taeubert et al., 2013). A good location in the river would improve chances of survival and reduce competition for nutrients (Taeubert et al., 2013). However, the longer the mussels stay on their host,

the probability that the host dies or gets eaten increases. At the same time, if multiple mussels all drop in the same spot, there could be an increased risk of predation and intraspecific competition. A prolonged excystment period can be seen as a strategy to reduce risk by bet hedging.

2.6 Conclusions

Our results strongly indicate that the duration of the parasitic phase of FPM has a significant effect on their post excystment performance. We found that juvenile mussels with the longest parasitic phase had a size, growth rate and survival advantage over those with the shortest one. Our results imply that post excystment fitness (performance) of the juvenile mussels most likely depends on parasite-host compatibility, and that temperature changes, for example due to climate change, can potentially affect the sensitive balance in this host-parasite interaction. Further research will allow us to identify the exact underlying factors that govern parasite-host compatibility.

3. Host (*Salmo trutta*) age influences resistance to infestation by freshwater pearl mussel (*Margaritifera margaritifera*) glochidia

Published: Janhavi Marwaha, Hans Aase, Juergen Geist, Bernhard C. Stoeckle, Ralph Kuehn, Per Johan Jakobsen (2019) Host (*Salmo trutta)* age influences resistance to infestation by freshwater pearl mussel (*Margaritifera margaritifera)* glochidia; Parasitology Research, 118 (5), 1519-1532.

3.1 Abstract

The freshwater pearl mussel (*Margaritifera margaritifera*) is an endangered bivalve with an obligate parasitic stage on salmonids. Host suitability studies have shown that glochidial growth and load vary significantly between host strains as well as among individuals of a suitable strain. Variation in host suitability has been linked to environmental conditions, host age and/or size, genetic composition of the host and parasite, or a combination of these factors. In our study we wanted to investigate if brown trout (*Salmo trutta*) displayed an age-dependent response to glochidial infestation. We hypothesised that 1+ naive brown trout hosts tolerate glochidial infestation better than 0+ hosts. In order to test our hypothesis, we infested 0+ and 1+ hatchery reared brown trout with glochidia from closely related mothers and kept them under common garden conditions. This allowed us to observe a pure age-dependent host response to infestation, as we eliminated the confounding effect of genotype-specific host interactions. We analysed the interaction between glochidial load and host condition, weight and length, and observed a significant age-dependent relationship. Glochidial load was negatively correlated to host condition in 0+ fish hosts, and positively correlated in 1+ hosts. These contradictory findings can be explained by a change in host response strategy, from resistance in young to a higher tolerance in older fish. In addition, we also examined the relationship between glochidial load and haematocrit values in the 1+ hosts, and observed that haematocrit values were significantly higher in heavily infested hosts. Our results have important

conservation implications for the management of wild pearl mussel populations, as well as for captive breeding programmes.

3.2 Introduction

The freshwater pearl mussel (FPM) *Margaritifera margaritifera* (also referred to as the pearlshell mussel in North America) is an endangered bivalve (Mollusc Specialist Group 1996; Araujo & Ramos 2000; Strayer et al. 2004) which has had a serious decline across its Holarctic range (Araujo & Ramos 2000; Machordom et al. 2003; Strayer et al. 2004; Geist 2010). This has made it the focus of several national and international conservation programmes (Araujo & Ramos 2000; Lopez-Lima et al. 2017). The FPM has a complex life cycle with an obligate parasitic stage on salmonids (Meyers & Millemann 1977; Young & Williams 1984b; Larsen 2005; Geist 2010; Taeubert et al. 2010; Taeubert & Geist 2017). Infective glochidia, released by gravid mothers, passively attach to a suitable fish host and become encysted on gills (Young & Williams 1984b; Wächtler et al. 2001; Taeubert et al. 2010; Taeubert et al. 2013) as parasites that depend on nutrient transfer from the host (Denic et al. 2015). In addition, they also reduce host swimming performance (Taeubert & Geist 2013). After 9–11 months (Larsen 2005), juvenile mussels excyst (May to June) and spend the next five years buried in the river sediment, after which they rise up to the substratum surface and develop into adults (Young & Williams 1984b).

The FPM is a specialist parasite which successfully metamorphoses only on the gills of Atlantic salmon (*Salmo salar*), sea trout (*Salmo trutta f. trutta*) and brown trout (*Salmo trutta f. fario*) in its European distribution (Young & Williams 1984b; Larsen 2005; Geist et al. 2006; Taeubert et al. 2010; Salonen et al. 2016; Taeubert & Geist 2017). Furthermore, some FPM populations were found to sometimes exclusively infest either *S. salar* or *S. trutta* even though both species are present in the river (Hastie & Young 2001; Karlsson et al. 2014; Österling & Wengström 2015; Ieshko et al. 2016; Salonen et al. 2017). It is assumed that *M. margaritifera* populations are best adapted to (historically) sympatric hosts as suggested from infestation

experiments (Taeubert et al. 2010; Salonen et al. 2017) as well as from similar genetic differentiation patterns among FPM and their hosts (Geist and Kuehn 2008); however contradictory evidence has also been reported regionally (Österling and Larsen 2013). Local adaptation of FPM has not yet been clearly demonstrated.

The parasitic glochidia are not selective in their attachment and they passively attach to all objects (even wood, plastic or paper) (Kat 1984; Dodd et al. 2005). Once attached to the gills of a suitable host, they induce an immune response and become encysted by gill epithelial cells (Nezlin et al. 1994). Glochidia that are unable to induce an immune response from their host will be shed off (Nezlin et al. 1996). On an unsuitable host, the cyst formed will be abnormal, causing the glochidia to die or be shed (Fustish & Milleman 1978; Kat 1984; Rogers-Lowery & Dimock 2006). Encystment is essential for the metamorphosis of glochidia into juvenile mussels (Haag 2012). It has been demonstrated that the cyst provides nutrition and mechanical protection to the glochidium (Arey 1932 a, b; Ziuganov et al. 1994; Wächtler et al. 2001; Denic et al. 2015). The host immune response is clearly essential for the glochidial metamorphosis into free living juveniles (Taeubert et al. 2010; Haag 2012; Taeubert & Geist 2017). In addition, the duration of the parasitic phase also influences size and post parasitic fitness of juvenile mussels (Marwaha et al. 2017). Juvenile mussels which had the longest parasitic phase had a size, growth rate and survival advantage compared with those with a short parasitic phase (Marwaha et al. 2017). Parasite-host compatibility is an important factor influencing glochidial load (glochidia per fish), growth and post parasitic performance of juvenile mussels.

Host suitability studies, wherein the most suitable hosts are identified, are an important focus in several conservation programmes. FPM-host suitability studies have shown that the most suitable host strains result in higher glochidial growth and glochidial load (Taeubert et al. 2010; Österling & Larsen 2013). Moreover, large individual host differences were observed with respect to glochidial growth and load among the suitable strains (Taeubert et al. 2010). The reason for individual differences among suitable hosts is not clearly understood. Bauer and Vogel (1987)

observed that glochidial development was related to their mortality on the host: glochidia developed faster on hosts with low glochidial loss. They proposed that these individual differences could be related to host immune response. In addition, individual host suitability could also be related to genetic composition of the host, host age, host condition, host size (length or weight), environmental conditions or a combination of several factors (Bauer & Vogel 1987; Taeubert 2014). Previous studies that have examined the relationship between host size (measured as either host weight or length) and age with FPM glochidial loads yielded several contradictory results.

Studies that have investigated the relationship between host size and glochidial loads have found positive correlations between host size and glochidial load (Young and Williams 1984b; Bauer & Vogel 1987; Hastie & Young 2001; Thomas 2011), negative correlations (Bauer 1987; Hastie & Young 2001) and no correlations at all (Cunjack & McGladdery 1991; Beasley 1996; Treasurer & Turnbull 2000; Hastie & Young 2001; Treasurer et al. 2006). The positive relationship between host size and glochidial load is believed to be transitory, becoming insignificant over time (Young & Williams 1984b; Bauer & Vogel 1987; Hastie & Young 2001; Thomas 2011). Larger fish initially have higher glochidial loads compared to smaller ones, probably as a result of larger gill surface area and higher ventilation rates (Young & Williams 1984b; Bauer & Vogel 1987; Hastie & Young 2001; Thomas 2011). However, a significant number of glochidia are lost in the first few months post infestation (Bauer & Vogel 1987, Hastie & Young 2001), and the positive correlation between host size and load becomes insignificant after the first week post infestation (Bauer & Vogel 1987). The decrease in glochidial loads in the following weeks is believed to be a result of the host mounting an immune response, and no correlation between host size and initial glochidial loads is observed thereafter (Meyers et al. 1980; Bauer & Vogel 1987; O'Connell & Neves 1999; Hastie & Young 2001). Most of these studies did not differentiate between the different host age classes and the observed results were mostly based on the relationship between host size and glochidial loads. Different host age classes can provide variable resources as well as differing immune responses to parasites (Izhar & Ben-Ami 2015).

Host age has generally been observed to be negatively related to glochidial load, and a decrease in glochidial loads with increasing host age has been observed in both wild (naturally infested) and hatchery-reared (artificially infested) hosts (Bauer 1987; Hastie & Young 2001). Typically in the FPM rivers, young wild salmonids are found to have the highest glochidial infestations (Awakura 1968; Karna & Millemann 1978; Bauer 1979, 1987; Young & Williams 1984b; Bauer & Vogel 1987), although older and larger host fish seem to be important in some northern European populations (Geist et al. 2006). Bauer (1987) observed a host age dependent relationship with glochidial mortalities; mortalities were higher in experimentally infested 1+ hosts compared to 0+ hosts, but this relationship was inversely density dependent in 1+ hosts. Age related differences, especially in wild hosts, were believed to be a result of a) reduced exposure of older hosts to glochidia due to behavioural differences (Hastie & Young 2001) and b) acquired immunity in older hosts as a result of previous glochidial infestations (Karna & Millemann 1978; Meyers et al. 1980; Bauer 1987; Bauer & Vogel 1987; Bauer et al. 1991; Ziuganov et al. 1994). In experimentally infested naive fish, as well as in wild hosts, age related differences could also be due to an age related immune response (Bauer 1987; Ziuganov et al. 1994; Hastie & Young 2001).

Although the relationship between host size, age and glochidial load has been investigated in several studies, it is difficult to disentangle the exact nature of the relationship between host size and age with glochidial loads. Moreover, some of the studies have used naturally infested wild fish which could lead to biased results due to the effects of acquired immunity from previous infestations (Bauer & Vogel 1987; O'Connell & Neves 1999; Rogers-Lowery et al. 2007; Thomas 2011; Chowdhury et al., 2018). In addition, it is likely that most of these previous studies have used glochidia with differing genotypes. Normally, in any host-parasite interaction, parasite success will depend on both the parasite and host genotypes, and their interaction (Carius et al. 2001; Lambrechts et al. 2005; Schmid-Hempel 2011; Barribeau et al. 2014). The presence of two or more parasitic genotypes could lead to competition for resources and hence higher levels of virulence on different host

genotypes (Taylor et al. 2005; Lagrue et al. 2011; Råberg 2014). These conditions would result in some glochidial genotypes being more successful compared to others on a single host, giving confounding results. Therefore, we believe that infesting naive fish hosts, of two different age classes, using glochidia with very similar genotypes will minimise the confounding effects of genotype-specific interactions. This will allow us to observe a host age dependent response to glochidial infestation under common garden conditions.

The main objective of our study was to evaluate the difference in host response to glochidial infestation among 0+ and 1+ naive fish hosts. We hypothesized that the host response to parasite infestation is dependent on the host's age, and the 1+ group will tolerate infestation better. In order to test our hypothesis, we used glochidia from closely related mothers to infest hatchery raised naive 0+ and 1+ fish. This allowed us to analyse the host response to infestation among the two age groups. We evaluated the relationship between glochidial load and host size (measured as weight, length and Fulton's condition factor) in our two host age groups, in order to identify a host age related difference in host response. In addition we also recorded Haematocrit (Hct) values in our 1+ hosts. Hct values (% red blood cells in blood volume) are positively related to glochidial infestations and are often used as a measure of respiratory stress caused by glochidial infestations (Meyers et al. 1980; Thomas et al. 2014; Filipsson et al. 2017).

3.3 Materials and methods

All experiments were carried out at the FPM rearing facility at Austevoll, Norway. The main water source for the rearing station comes from Lake Kvernavatnet, an oligotrophic lake with a size of 0.125 km^2 and a mean depth of 17.5 m. This water was used for maintaining the fish and adult mussels. It has a pH of 6.6, alkalinity of 0.108 mmol/l and the concentration of aluminium, iron, calcium, magnesium and nitrate as follows: Al – 180 µg/l; Fe – 200 µg/l, Ca – 4.2 mg/l, Mg – 1.8 mg/l, Na – 12 mg/l and Nitrat-N – 0.15 mg/l. The water was UV-treated and filtered through 30 µm mesh before use. The water temperature followed the natural fluctuation of the

lake and was between 5 and 17 ºC. Glochidial release and infestation of hosts occurred at an average water temperature of 16.2 ºC.

3.3.1 Glochidial collection and DNA extraction

Adult mussels (n=50) from the river Raudsjøbekken (Akershus County, Norway) were transferred to the FPM breeding station in June 2014. In a pre-screening, this mussel population was identified as one with very little genetic variation among individuals as revealed by analyses of nine microsatellites (data not shown). The mussels were kept in artificial rivers, with flowing water and fed with a diet of Shellfish® 1800 (Reed Mariculture Inc., Campbell, CA, USA) and Nanno 3600 (Reed Mariculture Inc.). Once the mussels started spatting in August, glochidial strings were collected and checked for maturation and viability (≥90%) using methods described by Watters and O'Dee (1999), before infesting the fish. Furthermore, 24 glochidia from each of the six randomly selected gravid mothers were analysed to confirm that they were genetically closely related.

A phenol-chloroform extraction was performed as described by Geist et al. (2008). Single and multiple glochidial samples were transferred into 1.5 ml eppendorf tubes and manually ground. For cell lysis, we added 500 µl lysis buffer (20 mM Tris pH 8.0, 5 mM EDTA pH 8.0, 400 mM NaCl, 1% SDS) and 25 µl Protinase K (10 mg/ml) to our samples and incubated them at 55 ºC for 12 hours. In order to separate the nucleic acids from the proteins and lipids, we added 600 µl (Roth) phenol/chloroform/isoamylalcohol (25:24:1) to our samples and centrifuged it. In order to precipitate the DNA, 500 µl of isopropanol was added to samples and they were centrifuged for 15 minutes. The DNA pellet was washed with 900 µl of 70% ethanol. Once the DNA pellet was dry it was dissolved in 50 µl of 5 mM Tris pH 8.5 and incubated at 55 ºC. Samples were then stored at -20 ºC for subsequent analyses.

We used nine microsatellite loci (MarMa2671, MarMa3050, MarMa3621, MarMa4143, MarMa4322, MarMa4726, MarMa5023, MarMa5167, MarMa5280) previously published by Geist et al. (2003) and Geist and Kuehn (2005). Analysis was carried out according to Geist and Kuehn (2005). Polymerase chain reactions

were carried out in a final volume of 12.5 µl with the following components: 25-50ng of genomic DNA, 200 nM of each primer, 0.2 mM of dNTP mix, 3 mM $MgCl_2$ (2 mM $MgCl_2$ for locus MarMa 5280), 1x PCR buffer and 0.25 U Taq (Solis Biodyne, Tartu, Estonia). The PCR was carried out on a Gradient thermal cycler (Eppendorf Mastercycler, Eppendorf, Germany) under the following cycling conditions: 94 ºC for 3 minutes followed by 35 cycles of 94 ºC for 30 seconds, 52-55 ºC for 30 seconds and 72 ºC for 30 seconds and a final extension at 72 ºC for 3 minutes. PCR products were separated on 5% denaturing 19:1 acrylamide/bisacrylamide gels on an ALFexpressII DNA analyser and scored with ALLELELINKS 1.02 software (Amersham Pharmacia Biotech, Amersham, UK). Electrophoresis was carried out with two internal standards (70 and 300 bp) in each lane. Additionally, an external standard (50–500 bp ladder) and a previously genotyped reference sample were included on each gel to standardize allele scoring and to facilitate cross-referencing among gels.

3.3.2 Fish infestations

We used naive hatchery reared brown trout (Botsvannsørret, *Salmo trutta*) obtained from the Statkraft facility in Eidfjord, Norway. Juvenile trout (0+) were transferred to the rearing station in July 2015 and kept in aerated 90 L tanks and fed until satiated. The experiment was done in two parts and ran over a period of two years (2015–2017). For the first part of the experiment we used 400 of the naive 0+ hosts (weight 2.3 ± 0.78 g, standard length 7± 0.66 cm). The remaining 500 naive 0+ hosts from the same batch (fish weight 9.8 ± 3.5 g, standard length 10 ± 1.17 cm) were allowed to grow for one year before being used in the second part of the experiment. For each part of our experiment, our test fish were all kept in a single tank and had no contact with glochidia pre and post infestation. To infest the fish, water levels in the fish tank was lowered and the fish were exposed to glochidia (500/L) for a period of 40 minutes with aeration. Glochidial strings collected from 16 mothers were used in the infestation baths. Fish samples (n=30) were taken out 48 hours post infestation to ensure that the fish were infested. Post infestation all infested fish were kept under equal food and ambient temperature conditions and fish mortalities were monitored. The temperature variation during the duration of our experiment did not vary significantly (Wilcoxon rank sum test: W = 69.5, p-value = 0.9076).

For the first part of the experiment we performed three controls over the infestation period, 60 days post infestation (dpi), 200 dpi and 300 dpi. At each control, 30 fish (at 60 and 200 dpi) and 70 fish (at 300 dpi) were randomly sampled and sacrificed. Fish were euthanised with an overdose of benzocaine (Benzoak Vet, ACD Pharmaceuticals) (exposure period of 10 minutes). Fish length and weight measurements were recorded to the nearest 0.5 cm and 0.1 g. We also recorded their infestation status (infested or uninfested) and, when infested, we counted the total number of glochidia (glochidial load) on one side of the fish. This was chosen randomly with a dice toss, with even and odd numbers deciding if all the left or right gill arches were used. In circumstances where no glochidia were found on one side of the fish, the other side was checked to confirm the infestation status of the fish. Glochidial load was estimated using the methods described by Dodd et al. (2005). Host gills were flushed thoroughly and the numbers of mussels on all gill arches were counted. Juvenile mussel mean size was also recorded by measuring the length of the widest part of the mussels to the nearest 0.1 μm. For the second part of the experiment (1+ hosts), we only recorded fish length and weight measurements and glochidial load at 300 dpi. The Fulton's condition factor was calculated using the formula $CF = 10^5 * W/L^3$ where W is the weight in grams and L is the total length in centimetres (Morton and Routledge 2006; Davidson et al. 2009).

In addition, we measured the haematocrit values of the 1+ fish only, because of the difficulty involved in collecting adequate blood samples from the 0+ hosts. Haematocrit (Hct) values (% red blood cells in blood volume) can be used as a measure of the oxygen carrying capacity of blood in fish (Gallaugher and Farrell 1998). Blood samples (1 ml) were taken from the caudal vein using Venojec vacutainer 3 ml syringes coated with Li-heparin and Venojec multisample 20G fitted with 0.9 × 40 mm needles. Blood samples were centrifuged in 100 μL microcapillary tubes at 13000 rpm for 10 minutes in a Hettich Haematocrit centrifuge and Hct was calculated as the percentage of red blood cells of centrifuged samples.

3.3.3 Statistical analysis

We used the statistical package R, version 3.4.3 (R Core team, 2017) for our analysis. We compared the difference in host Fulton's condition factor, weight and standard length (will be referred to as host traits), glochidial load and juvenile mussel mean size, between and within our host age groups. We also compared the difference in glochidial load between the two host age groups by standardising glochidial load by host weight (number of glochidia/gram fish weight). To do this we used either a Kruskal Wallis test or ANOVA, depending on whether the data fulfilled conditions of normality. For Hct values, we subdivided the 1+ infested fish in three groups; high (200+ glochidia, on one side), medium (1-199 glochidia, on one side) and uninfested, and then compared the Hct values among these groups. Correlation tests (Spearman's or Kendall Tau) were used to check correlations between all our test variables. We used a generalised linear mixed effect model (GLMM) with penalized quasi-likelihood approach and Gaussian as the family, to examine the relationship between glochidial load and host traits. We used the glmmPQL function from the MASS library in R, with glochidial load as the predictor variable, host traits as the response variable and individual hosts as random factor to bring in the heterogeneity among hosts. The same result was obtained if heterogeneity among hosts was not considered. We used a linear regression model to test the relationship between the glochidial load and juvenile mussel mean size and used mean size as the response variable and glochidial load, condition factor and the interaction between them as covariates. We performed the above analysis using the R library leaps. Since only glochidial load was found to be significant, we performed a linear regression with mean size versus glochidial load. For Hct values we used a generalised linear model with Gaussian as the family to examine the effect of glochidial load.

In order to verify whether the six adult pearl mussel mothers were closely related, glochidia of two randomly selected mothers were pooled since the computational approach of pairwise analysis of genetic divergence requires more than four individuals per group. This grouping (three groups with eight glochidia each) was used for all subsequent population genetic computations. For each group allele frequencies, average allele numbers per locus (A), expected and observed

heterozygosities (HE, HO) were calculated with GENEPOP 4.0 (Rousset 2008). The same software was used to test the loci for genotypic disequilibrium, to test for significant population differentiation among all pairs of populations using 100,000 iterations and 1000 dememorisation steps (Raymond and Rousset 1995), and to test each locus in each population for conformance with Hardy–Weinberg (HW) expectations. Group pairwise analyses of genetic divergence (Jost's Dest), which measures the fraction of allelic variation among populations was calculated with the software GENALEX (Peakall and Smouse 2006). Each microsatellite locus was assessed for the presence of null alleles and genotyping errors using MICROCHECKER v.2.2.3 (van Oosterhout et al. 2004).

3.4 Results

Glochidial load was significantly higher in the 1+ fish hosts (mean 212.79 glochidia/fish) compared to 0+ (4.47 glochidia/fish) (Kruskal test: chi-squared = 75.458, df = 1, p-value = <2.2e-16). This remained significant even when glochidial load was standardised by host weight (number of glochidia/gram fish) (Kruskal test: chi-squared = 15.899, df = 1, p-value = 6.68e-05; Figure 3.1). Mean juvenile mussel sizes did not vary between the two host age groups (ANOVA: Std. Error = 0.0085, t-value = 0.963, p-value = 0.338).

3.4.1 0+ hosts

We did not observe any differences in Fulton's condition factor (Kruskal test: chi-squared = 0.0291, df = 1, p-value = 0.865), host weight (Kruskal test: chi-squared = 0.7376, df = 1, p-value = 0.514) or standard length (ANOVA: Std.Error = 2.4949, t-value = 1.201, p-value = 0.232) between the infested and uninfested hosts at 300 dpi. However, a significant negative correlation was observed between glochidial load and host weight (Kendall Tau: τ = -0.288, p-value = 0.0006), standard length (Kendall Tau: τ = -0.256, p-value = 0.003) and a moderately significant one with Fulton's condition factor (Spearman's: ρ = -0.2051, p-value = 0.081) among the infested 0+ hosts. The GLMM model also showed a significant negative relation between glochidial load and Fulton's condition factor (glmmPQL: Estimate = -0.006,

Std.Error = 0.0032, t-value = -1.891, p-value = 0.062; Figure 3.2), host weight (glmmPQL: Estimate = -0.281, Std.Error = 0.094, t value = -2.995, p-value = 0.0038) and host standard length (glmmPQL: Estimate = -0.0806, Std.Error = 0.033, t-value = -2.44, p-value = 0.0172). We did not observe any significant correlations between host traits and glochidial loads at 60 dpi (Kendall Tau: Fulton's condition factors: τ = 0.0186, p-value = 0.887; weight: τ = -0.0238, p-value = 0.857, standard length: τ = -0.127 , p-value = 0.334) and at 200 dpi (Spearman's: Fulton's condition factors: ρ = -0.1337, p-value = 0.481; weight: ρ = -0.01, p-value = 0.956; standard length: ρ = 0.0599, p-value = 0.7531).

3.4.2 1+ hosts

There was a significant difference in Fulton's condition factor between the infested and uninfested hosts (ANOVA: Std.Error = 0.018, t-value = -4.038, p-value = 0.0001, Figure 3.3A). This became even more significant when comparing the highly infested and the uninfested groups (ANOVA: Std. Error = 0.0189, t-value = -6.039, p-value = 2.73e-07, Figure 3.3B). We observed a significant positive correlation between glochidial load and Fulton's condition factor (Spearman's: ρ = 0.3054 p-value = 0.0368) and the generalised linear model also showed a significant positive relationship between these variables (glmmPQL: Estimate = 1.251e-04, Std.Error = 4.864e-05, t-value = 2.572, p-value = 0.0135, Figure 3.4). In addition, a significant positive correlation between host weight (Spearman's: ρ = 0.3968, p-value = 0.06) and host standard length (Spearman's: ρ = 0.4052, p-value = 0.055) in the high infestation group was also observed. Juvenile mussels were larger on the high infestation group compared to the medium group (ANOVA: Estimate = -0.0167, Std.Error = 0.0085, t value = -1.963, p-value = 0.0559, Figure 3.5A). A significant positive correlation was also observed between mean juvenile mussel size and glochidial load (Kendall Tau: τ = 0.2893, p-value = 0.0043; LM: Std.Error = 1.932e-05, t-value = 2.408, p-value = 0.02, Figure 3.5B).

Hct values did not differ between infested and uninfested fish groups. However, Hct values of the high infestation group were significantly higher than the medium infested and uninfested groups (Medium: Kruskal-Wallis: chi-squared = 4.6055, df =

1, p-value = 0.0318; Uninfested: Kruskal-Wallis: chi-squared = 5.2263, df = 1, p-value = 0.022, Figure 3.6A). Rank correlation tests showed a significant correlation between glochidial load and Hct values (Spearman's: ρ = 0.3312, p-value = 0.0299), however the GLM model showed only a moderately significant relationship between these variables (GLM: Estimate = 0.134e-05, Std.Error = 5.291e-05, t-value = 1.726, p-value = 0.0912, Figure 3.6B).

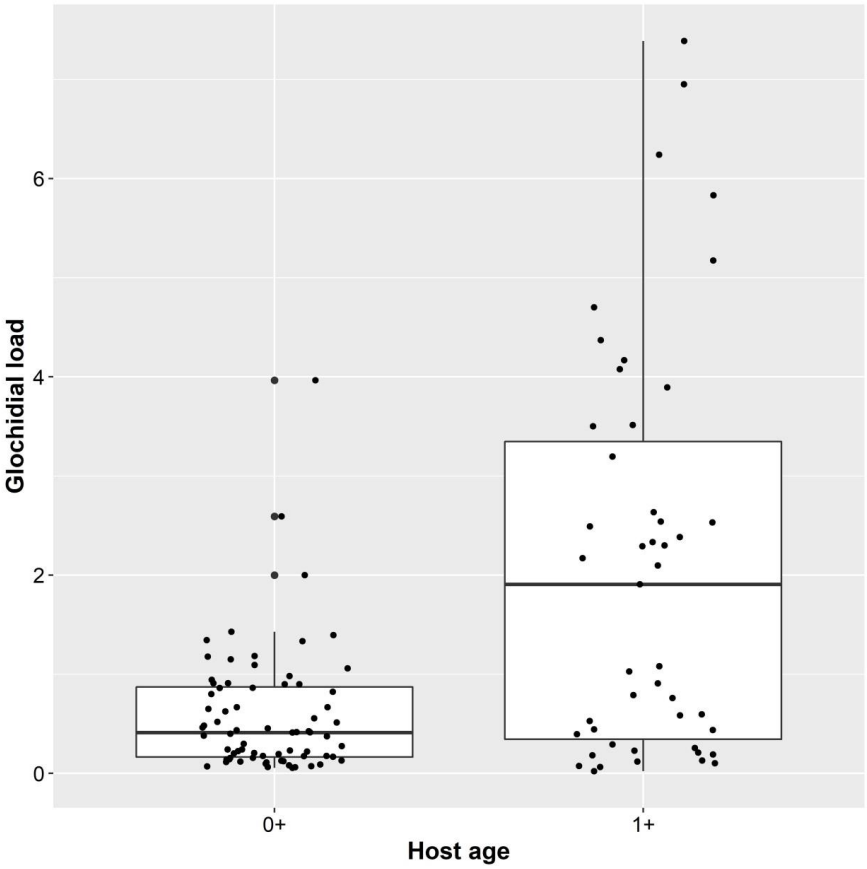

Figure 3.1: Box plot showing the difference in glochidial abundance (normalised by fish weight) in the 0+ (n = 72) and 1+ (n = 50) fish hosts. The thick line displays the median, the boxes show the 25 and 75% quartiles, and the whiskers show the range of the dataset. The dots show the individual data points.

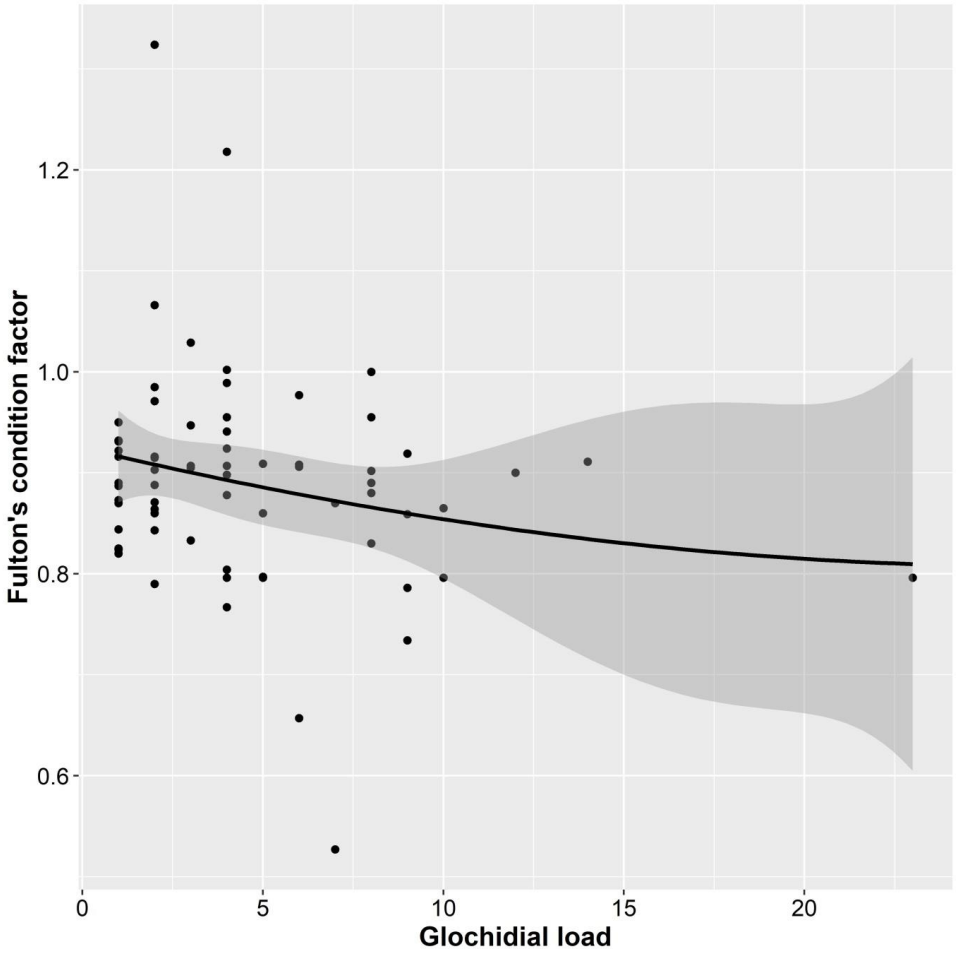

Figure 3.2: Relationship between glochidial load and Fulton's condition factor in 0+ fish hosts. The thick black line represents the cubic smoothing spline and the 95% confidence intervals are in grey.

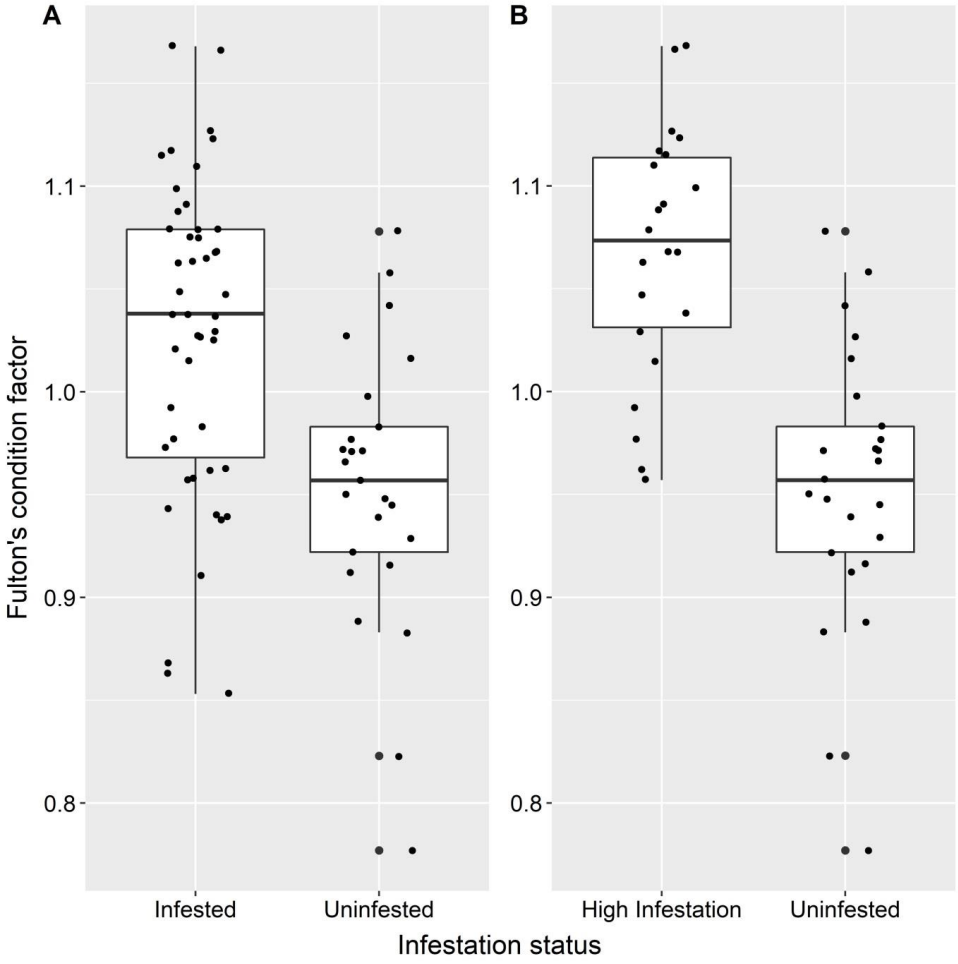

Figure 3.3: Difference between the Fulton's condition factor between the A) Infested and uninfested 1+ hosts and B) High infestation group (200+) and uninfested 1+ groups. The thick line displays the median, the boxes show the 25 and 75% quartiles, and the whiskers show the range of the dataset. The dots show the individual data points.

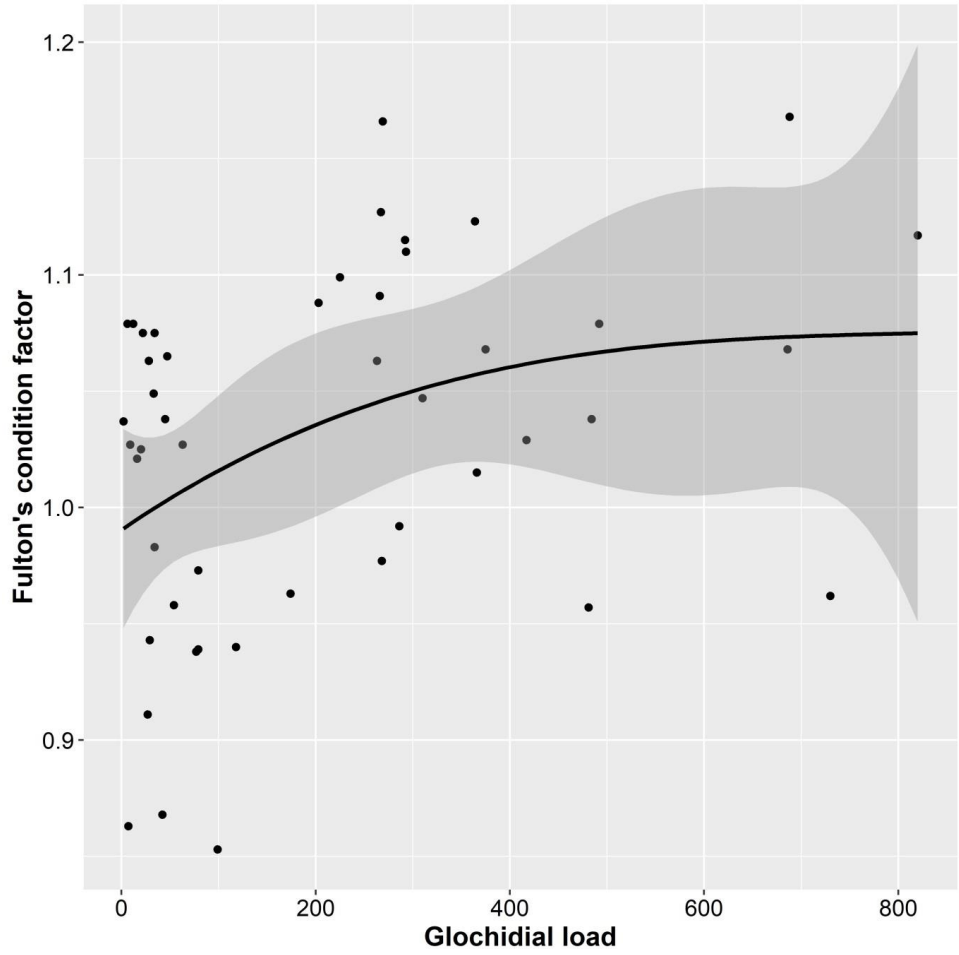

Figure 3.4: Relationship between the glochidial load and Fulton's condition factor in 1+ hosts. The thick black line represents the cubic smoothing spline and the 95% confidence intervals are in grey.

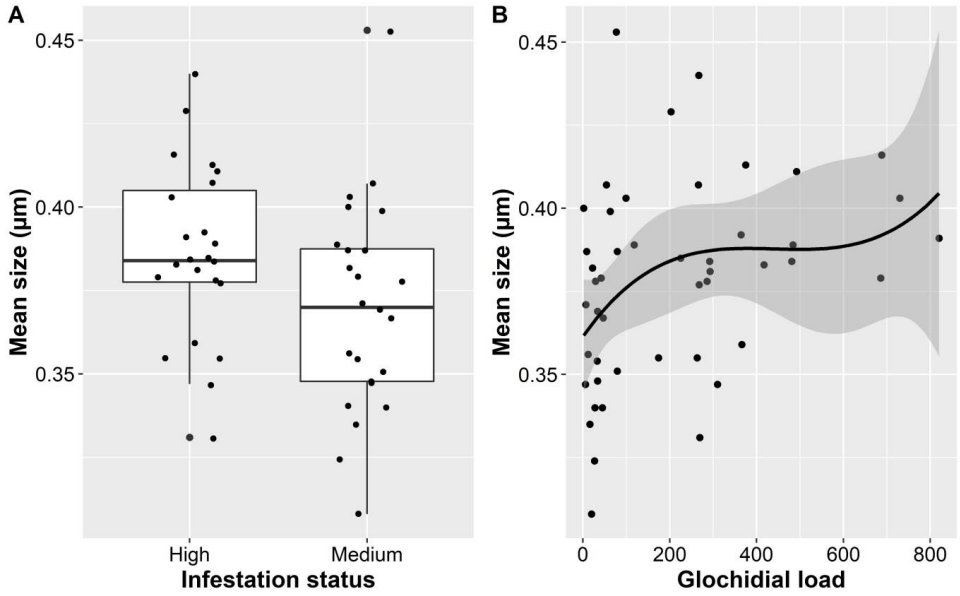

Figure 3.5: A) Differences in juvenile mussel mean size (µm) between the 1+ high infestation and medium infestation host groups. The thick line displays the median, the boxes show the 25 and 75% quartiles, and the whiskers show the range of the dataset. The dots show the individual data points. B) Relationship between glochidial load and juvenile mussel mean size in 1+ hosts. The thick black line represents the cubic smoothing spline and the 95% confidence intervals are in grey.

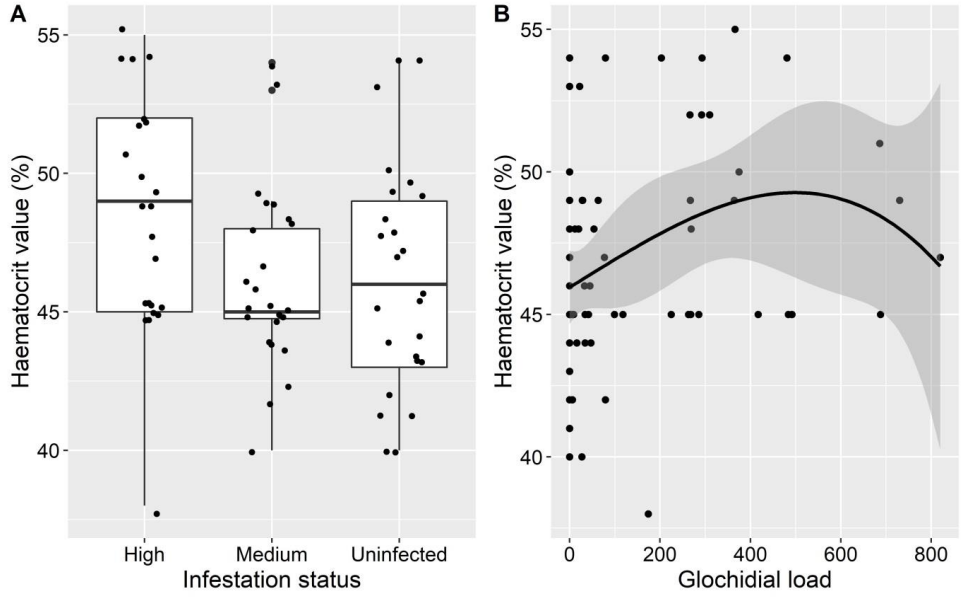

Figure 3.6: A) Differences between the Hct values between the high, medium and uninfested 1+ hosts. The thick line displays the median, the boxes show the 25 and 75% quartiles, and the whiskers show the range of the dataset. The dots show the individual data points. B) Relationship between glochidial load and Hct values. The thick black line represents the cubic smoothing spline and the 95% confidence intervals are in grey.

3.4.3 Microsatellite analysis

The test for the presence of null alleles and genotyping errors using MICROCHECKER v.2.2.3 (van Oosterhout et al. 2004) revealed null allele frequencies below 0.2. Since this level has been shown to have very little impact on population delineation and divergence estimates (Dakin and Avise, 2004; Carlsson 2008) all loci were included. The test for genotypic disequilibrium for each pair of the nine polymorphic microsatellite loci over all populations gave no significant value and no significant deviations from the expected HW proportions were observed after applying sequential Bonferroni correction.

Microsatellite diversity varied among loci, with one allele at MarMa2671, MarMa4322, MarMa5023 and five alleles at locus MarMa4143. Allelic richness (adjusted for sample size) ranged from 1.5 alleles per locus to 1.7 alleles per locus. Levels of observed and expected heterozygosity varied hardly between groups ranging from 0.100 to 0.167 and from 0.162 to 0.188, respectively. Glochidia of the three groups were closely related reflected by Jost's distance (Dest (mean) = 0.040; SD = 0.033).

3.5 Discussion

The results of our study show that host response to glochidial infestation was dependent on the host age under equal food and temperature conditions, and under the condition that none of the hosts had experienced previous contact with glochidia. Host condition of the infested 0+ fish hosts had a negative correlation with glochidial load, whereas a strong positive one in the 1+ hosts was evident. In addition, the Hct values were significantly higher only in the heavily infested 1+ hosts. With minimal variation in the infecting glochidial genotypes, our results show a clear host age dependent response to glochidial loads.

Age related differences in host susceptibility and immune response to glochidial infestation have been observed in some FPM glochidia-host studies (Bauer 1987; Bauer & Vogel 1987; Hastie & Young 2001). The reasons suggested for these differences were dissimilarities in gill morphology and the chemical composition of host gill mucus and blood (Young et al. 1987; Hastie & Young 2001). However, Karna and Millemann (1978) did not find any evidence of this. Nevertheless, evidence from several studies shows that FPM glochidia-host interaction, i.e. successful glochidial encystment, survival and metamorphosis into juveniles, is highly dependent on the parasite-host compatibility which in turn depends on host immune response (Nezlin et al. 1996; Haag 2012). In the first few weeks post infestation, the host generally loses a significant number of glochidia. This loss occurs due to a tissue response in the first seven days post infestation, and in the following weeks thereafter, due to a humoral response (Bauer 1987; Bauer & Vogel

1987). As previously mentioned, Bauer (1987) observed an age dependent difference in glochidial mortalities in *S.trutta* hosts and proposed that these were a result of age related differences in host immune response to glochidial infestation. He suggested that 0+ hosts had a weaker immune response to infestation, but this was density dependent, i.e. it increased with increasing glochidial density. The 1+ hosts displayed a stronger immune response which was inversely density dependent, i.e. the immune response was especially strong when the glochidial loads were low. In our experiment we used host condition as a measure of host response to glochidial infestation, and we observed a positive relationship between glochidial load and host condition in the 1+ hosts, and a negative one in the 0+ hosts. The 1+ hosts with the highest glochidial loads also had the best condition when compared with the uninfested and medium infested hosts. In accordance with Bauer's (1987) proposal, our results also indicate that the 1+ hosts mobilised a weaker immune response when glochidial loads were high. This probably resulted in enhanced growth in the heavily infested hosts. To the best of our knowledge, this is the first evidence of a positive effect of mussel glochidia on a fish host whose survival probability increases with size. The 0+ hosts with the highest glochidial loads had the lowest condition, suggesting a strong immune response which was density dependent.

Bauer (1987) and Bauer and Vogel (1987) observed that glochidial development (size) was dependent on glochidial mortality on the hosts, i.e. glochidia were larger on fish with low glochidial mortalities and vice versa. The authors proposed that a weak host immune response would result in lower glochidial mortalities and would provide the developing glochidia with conditions conducive for glochidial development and growth. This would result in larger glochidia. Moreover, glochidial growth has also been reported to be positively related to host condition; i.e. hosts with a good condition provide higher energy resources for the developing larvae (Österling & Larsen 2013). In our study, we observed a significant positive relationship between the mean size of juvenile mussels and glochidial load in the 1+ hosts, i.e. heavily infested 1+ hosts with the best condition had larger juvenile mussels. However we did not observe any correlation between juvenile mussel size and glochidial load in the 0+ hosts. We propose that a weaker immune response in the heavily infested 1+ hosts

provided the glochidia with conditions beneficial for their growth and development, resulting in larger juveniles on heavily infested 1+ hosts compared to medium infested ones. Our observations also support our proposal that the 1+ hosts mounted a weaker immune response compared to the 0+ hosts. In our experiment we did not specifically examine the differences in immune response in our two age groups. Also, individual differences in growth of hosts could be a possible reason for observed differences in host condition at the end of the experiment. Nevertheless, based on evidence from previous studies (Bauer 1987, Bauer & Vogel 1987), in addition to the importance of host immune response in the FPM glochidia-host interaction, we believe that the difference in the relationship between glochidial load and condition factor we observed between the two host age groups is related to a difference in the immune strategy employed by them.

Two host defence strategies have been described in the literature: i) resistance, which is the ability to prevent or reduce a given parasite, and ii) tolerance, which is the ability to limit the damage caused by a given parasite (Råberg et al. 2009; Best et al. 2014; Jackson et al. 2014; Råberg 2014; Klemme & Karvonen 2016; Kutzer & Armitage 2016; Adelman & Hawley 2017). An important prediction of the life-history of an organism is that optimal energy allocation is towards important traits such as growth, maintenance and survival (Sandland & Minchella 2003; Šimková et al. 2008). Under natural circumstances, hosts would typically have a limited access to resources, and resource allocation towards an optimally functioning immune system and/or an effective immune response would be costly for the host (Sheldon & Verhulst 1996; Norris & Evans 2000; Martin II et al. 2003). Moreover, resource allocation towards an effective immune response can be influenced by the age, sex and life history stage of the host, and also by environmental or ecological factors that can have an effect on the physical condition of the host (Wilson et al. 2002; Sandland & Minchella 2003; Hämäläinen et al. 2015, Klemme & Karvonen 2016). Thus, the immune defence strategy employed by hosts can vary with age and younger hosts are generally expected to invest more in a stronger immune response (Poulin 1993; Thomas et al. 2000; Jackson et al. 2014). When a host is faced with the risk of

parasitism, there could either be a higher investment in immune defence at the expense of other traits such as growth or reproduction, or a trade-off between resource allocation towards growth and an expensive immune response (Gustafsson et al. 1994; Nordling et al. 1998; Siva-Jothy et al. 1998; Veiga et al. 1998; Moreno et al. 1999; Ilmonen et al. 2000; Bonneaud et al. 2003; Soler et al. 2003; Brommer 2004; Jacot et al. 2004; Ahtiainen et al. 2005; Tschirren & Richner 2006; Lefevre et al. 2008 Šimková et al. 2008). Sometimes a trade-off between an expensive immune response and growth could be advantageous to the host since an effective immune response can also lead to damage to host tissue (Klemme & Karvonen 2016). In most natural circumstances, host defence would be a combination of the two defence strategies (Jackson et al. 2014).

Host tolerance to parasitic infestation has been measured as the relationship between host condition and parasitic load (Jackson et al. 2014). There are several examples in the literature where this relationship was positive for parasite infested individuals. For example, an increase in growth and/or improved body condition has been observed in fish hosts infected by plerocercoids of *Schistocephalus solidus* (Milinski 1985, Arnott et al. 2000), *Ligula intestinalis* (Museth 2001, Loot et al. 2002) and *Posthodiplostomum cuticola* (Ondrackova et al. 2004). The reason for an increase in host weight or an improved condition could be related to a change in fish foraging behaviour, food conversion efficiency and reduced activity, or a combination of these factors (Arnott et al. 2000). Fish infested with glochidial parasites have been reported to have reduced activity and they also become less bold (Thomas 2011; Horký et al. 2014). This is believed to be a result of the physiological impact of glochidia on host gills, leading to respiratory stress and thus reduced movement (Thomas 2011; Horký et al. 2014). We believe that reduced movement, which will conserve energy, in addition to ad libitum feeding will result in improved host condition. Moreover, the higher host condition observed in heavily infested 1+ hosts, compared to the medium and uninfested groups, despite all hosts being fed ad libitum, clearly indicates that heavily infested hosts invested more resources in growth due to high glochidial infestation.

In contrast to the 1+ hosts, we did not observe any difference in the host condition between infested and uninfested 0+ hosts. In addition, we observed a negative relationship between host traits and glochidial loads. We believe that the small size of the 0+ hosts, and in turn less resources, led to a resistance strategy. It is believed that younger hosts ideally invest more in fighting parasites to ensure future reproductive success, compared to older hosts (Poulin 1993). Host resistance or tolerance to infestation is believed to be influenced by host age and/or sex, genetic components of the immune system and environmental factors (Råberg 2014; Kutzer and Armitage 2016). Jackson et al. (2014) investigated the age-dependent physiological mechanisms influencing host tolerance to parasite infestations in male voles. They measured the expression of immunity genes (Gata3) in different age classes to observe if this explained variation in tolerance. Mature voles were observed to be less resistant to parasites compared to immature ones, and a positive relationship was also observed between host age and parasite numbers. The age-dependent difference in tolerance was mirrored by an increase in the expression of Gata3, i.e. it increased with parasite load in adult voles and vice versa. The underlying genetic or physiological mechanisms that influence host age-dependent tolerance or resistance are not yet clearly understood and further studies are required.

Haematocrit values, which represent respiratory stress as a result of glochidial infestation in host fish, were significantly higher in the 1+ hosts which were infested with 200+ glochidia (on one side) compared to those with moderate intensities (1-199, on one side) and uninfested hosts. We also observed a positive correlation between Hct values and glochidial loads. Although we were unable to measure Hct values in the 0+ hosts, nevertheless, our observations give a clear indication that glochidial loads exceeding 200 glochidia per fish (on one side) resulted in respiratory stress and hence a compensatory increase in Hct values. High glochidial loads are typically associated with reduced critical swimming speed in trout, which affects the oxygen requirements for a specific activity or reduces the oxygen uptake due to damaged gills (Taeubert & Geist 2013; Filipsson et al. 2017). Moreover, Filipsson et al. (2017) observed that glochidiosis affects host metabolic rates and oxygen carrying

capacity, and the resulting compensatory increase in Hct levels was believed to enhance oxygen transport capacity of the host. The increase in Hct levels was explained by the increase in the mean corpuscular volume and decrease in the mean corpuscular haemoglobin concentration (Meyers et al. 1980; Thomas et al. 2014; Filipsson et al. 2017). Although low glochidial loads are not believed to have a harmful effect on salmonid performance (Treasurer et al. 2006; Taeubert & Geist 2013), Thomas et al. (2014) observed that fish with glochidial intensities of just 1–204 glochidia per fish took a longer time to reach the basal ventilation rate after a stressor. Glochidial intensities in our experiment ranged between 200–820 glochidia (on one side) in our heavily infested hosts and the elevated Hct values clearly indicate a compensatory response as a result of high glochidial infestation.

The results from our study show clear differences in host age dependent response to glochidial infestation. This can be explained by a change in host response strategy from sensitivity in young to tolerance in older fish. We propose that the fish host is an important filter for glochidial attachment and metamorphosis. The results from our experiment are important in the context of developing optimal strategies for conserving endangered freshwater pearl mussel populations and their host fish in the wild, as well as in captive breeding programmes. For instance, naive 1+ hosts were the most suitable hosts and should be preferentially used in captive breeding to minimize possible selection and drift effects, as well as to maximize the production of young mussels. Moreover, our observations also indicate that glochidial loads which were within the recommended range on a host fish (5-100 per gram fish) (Taeubert and Geist 2013), resulted in respiratory stress, as indicated by the higher Hct values in heavily infested hosts. Since glochidial development and successful metamorphosis into juvenile mussels is highly dependent on good host condition and survival, conservation efforts should focus on methods that can guarantee this (Taubert and Geist 2013, Filipsson et al. 2017). Artificial infestation programmes should ensure low infestation rates on hosts, as this can ensure the well-being and survival of infested fish that are released into streams, which in turn will promote successful release of juvenile mussels (Taubert and Geist 2013, Filipsson et al. 2017). The pearl mussel salmonid parasite-host system is a unique system which involves the

interaction between a very long-lived specialised parasite that can infest a host with a much shorter life span. This provides a particularly interesting system in which eco-evolutionary strategies can be identified.

5. General Discussion

"*The extinction of a species, each one a pilgrim of four billion years of evolution, is an irreversible loss. The ending of the lines of so many creatures with whom we have travelled this far is an occasion of profound sorrow and grief. Death can be accepted and to some degree transformed. But the loss of lineages and all their future young is not something to accept. It must be rigorously and intelligently resisted."*
– Gary Snyder in Practice of the Wild (1990).

An important prerequisite for the conservation of an endangered species is a comprehensive knowledge of its biology and ecology. The obligate parasitic phase in the *M. margaritifera* life cycle necessitates the understanding of host-parasite interactions and factors that influence them, because this will help to develop robust conservation strategies.

The results of this study reveal novel insights into the relationship between *M. margaritifera* and their salmonid hosts, and they strongly emphasize the importance of host-parasite compatibility. Compatibility depends on host-parasite interactions, which are governed by host factors (species, age), parasite factors (population, glochidial load, virulence) and environmental factors (temperature). Figure 5.1 enumerates these, highlighting (in green) the ones addressed in this study. The results also strongly indicate that the host immune response and the genetic composition of both host and parasite also govern the outcome of the host-parasite interaction.

In the first experiment, the duration of the parasitic phase was found to have a positive influence on the post parasitic fitness of juvenile mussels (Chapter 2). Juvenile mussels that had a longer parasitic phase had a size, growth and survival advantage compared to those with a shorter one. These results were observed across eight *M. margaritifera* populations, which suggests a general trend. In addition, temperature was identified as an important factor governing excystment, with higher temperatures decreasing the duration of the parasitic phase. In the second experiment,

Salmo trutta, infested with glochidia from closely related mothers, displayed an age-dependent immune response to glochidial infestation (Chapter 3). The older 1+ hosts were more tolerant to glochidial infestation compared to the younger 0+ hosts, as shown by their differing condition factors. In the same experiment, glochidial loads exceeding 200 glochidia per fish were found to result in elevated haematocrit values. In the third experiment, differential glochidial virulence (measured as host mortality) was observed on the two salmonid host species, with higher virulence seen in the less suitable brown trout host (Chapter 4). This experiment also revealed that most individual mothers were able to infest both salmonid host species. However, they did not infest both host species with equal probability (some displayed a bias towards either of the two hosts). In line with previous results, the average glochidial load (number of glochidia per fish) was observed to be the highest on the most suitable salmonid host species.

5.1 Host-parasite compatibility

In Chapter 2, the post parasitic fitness of juvenile mussels was dependent on the duration of their parasitic phase, i.e. juveniles with the longest parasitic phase excysted at a larger size and had higher growth and survival rates. These juvenile mussels would most probably have better resources to start their benthic existence, and hence have better survival (Österling & Larsen, 2013; Eybe et al., 2014). Moreover, and in line with other studies, glochidial fitness (abundance, prevalence, size) varied among salmonid host species, individuals of the principal salmonid species, as well as different age groups of the principal species (Chapters 3 and 4) (Taeubert et al., 2010; Salonen et al., 2017; Taeubert & Geist, 2017; Clements et al., 2018). This variation in glochidial fitness is believed to be a result of host-parasite compatibility (Haag, 2012). It is very likely that juvenile mussels that excyst early have low compatibility with their hosts. As a result, they are not as well developed as those that excyst late. However, there is also most likely an optimal time for mussel excystment in relation to water temperature. This means that at the ideal temperature, the maximum numbers of mussels will excyst.

It is well established that the parasitic phase is essential for glochidial development and metamorphosis into juveniles, because the host (cyst) provides nutrition and mechanical protection to the developing glochidium (Arey 1932a, 1932b; Ziuganov et al., 1994; Wächtler et al., 2001; Denic et al., 2015). Therefore, it is likely that the degree of compatibility with the host fish will influence how successfully the host builds the 'house' cysts around the glochidia, which in turn affects the establishment and degree of nutrition available to the developing glochidia. Therefore, host species and individual hosts of a suitable species vary in terms of the conditions they provide for the developing glochidia. This variation is a result of host-parasite interactions (discussed below) which influence the degree of host-parasite compatibility (Taeubert et al., 2010).

The pearl mussel population tested in the third experiment was reported to use Atlantic salmon as its principal host (Johnson et al., 2008), and the experiment reaffirmed it. The high degree of compatibility between the glochidia and the Atlantic salmon host indicates that the parasite is well adapted to this host. The host itself is probably tolerant to this glochidial infestation, and hence could be said to have a weak influence on the interaction outcome. Glochidia are short-term parasites, and leave their hosts at the end of their developmental period. It is more likely that a host develops tolerance to a parasite that leaves (when coliving does not have a mortal effect), as opposed to a parasite that never leaves, in which case resistance is the expected host response.

This thesis adopts a biased view of host-parasite compatibility, as it focuses primarily on the parasite's fitness when considering compatibility. Glochidia have a negative effect on their hosts (discussed in section 5.3), and high compatibility may not always have a positive influence on the host's fitness. In Chapter 4, however, the results do show a higher survival of compatible infested fish. In other words, in this case host-parasite compatibility was favourable for the most compatible host.

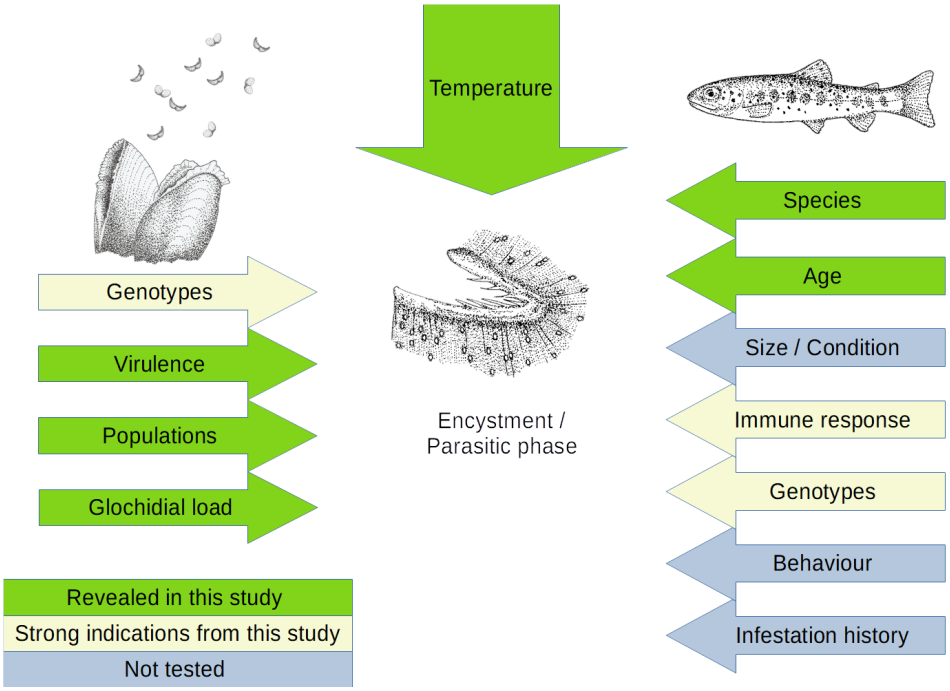

Figure 5.1: Host, parasite and environmental factors that have an influence on the *M. margaritifera* salmonid host-parasite interaction. Illustration by Ragnhild Aakre Jakobsen.

5.2 Factors that influence host-parasite interactions

In this section, factors that influence host-parasite interactions, and that are revealed in the study, are discussed in detail.

5.2.1 Temperature

Water temperature was identified as an important cue for juvenile mussel excystment, with higher temperatures decreasing the duration of the parasitic phase. As reproduction and development of *M. margaritifera* is dependent on water temperatures (Hastie & Young 2003a; Skinner et al., 2003; McIvor & Aldridge, 2008; Österling et al., 2008; Taeubert et al., 2013), any change in the natural temperature regime (e.g. due to climate change) can affect the sensitive relationship between parasite and host. Although the data revealed that temperature was an

important factor influencing juvenile mussel excystment, it did not explain why the post excystment growth, under equal temperature conditions, was different. The duration of the parasitic phase was clearly dependent on either the temperature at which they develop, compatibility with the host, or both (Lefevre & Curtis, 1912; Ziuganov et al., 1994; Taeubert et al., 2010; 2013; 2014).

5.2.2 Host age

Host age was revealed to be an important factor that determined the host immune response to glochidial infestation; i.e. resistance in 0+ hosts and tolerance in 1+ hosts. Moreover, the 1+ tolerant hosts had higher glochidial loads compared to the 0+ resistant hosts, even when it was standardised by host weight (number of glochidia/gram fish weight). Naive 0+ hosts are believed to be the most suitable hosts, and are therefore most commonly used in artificial rearing programmes (Eybe & Thielen, 2010; Thomas et al., 2010). However, these novel results show that naive 1+ hosts are the most suitable hosts for *M. margaritifera*. Because they are tolerant to glochidial infestation, they do not pay the cost of resistance.

In the experiment, hosts were infested with glochidia from closely related mothers (i.e. nearly genetically identical glochidia) in order to eliminate the confounding effects of parasite genotype-specific interactions. The age-dependent host resistance is probably associated with the life history strategy of the host. The immune defence strategy employed by hosts can vary with age, and younger hosts are generally expected to invest more in a stronger immune response compared to older hosts (Poulin, 1993; Thomas et al., 2000; Jackson et al., 2014). However, the strong immune response or resistance displayed by a host is associated with a fitness cost, and this cost will be dependent on both the mechanism of resistance and the environment of the host (Koskella, 2018). If the cost of a strong immune response (resistance) outweighs its benefit, then the host has to bear a net fitness cost. This could be, for example, reduced host condition as observed in this study, reduced survival, or reduced reproduction (Rigby et al., 2002). Usually in nature, fish hosts have limited access to resources, and resource allocation towards an optimally functioning immune system and/or an effective immune response would be costly for

the host (Sheldon & Verhulst, 1996; Norris & Evans, 2000; Martin II et al., 2003). Therefore, allocation of resources towards an effective immune response can be influenced by the host age, sex, life history stage, environmental or ecological factors, and can have an effect on the physical condition of the host (Wilson et al., 2002; Sandland & Minchella, 2003; Hämäläinen et al., 2015; Klemme & Karvonen, 2016). As the experiment was performed under common garden conditions, it is likely that the results show the influence of host age on the interaction outcome. In addition to age, other factors could also lead to individual differences in host compatibility in nature. These factors could be host size, relative gill surface area, ventilation rates, host immunological and physiological conditions, behaviour, infestation history and the presence of other parasites (Karna & Millemann, 1978; Young & Williams, 1984b; Bauer & Vogel, 1987; Hastie & Young, 2001; Thomas, 2011; Österling & Larsen, 2013; Wengström et al., 2016). Furthermore, it could be speculated that glochidial virulence varies among the two host age groups. For example, glochidia may penetrate deeper into the relatively smaller gills of the 0+ hosts, resulting in higher gill damage, and therefore resulting in higher virulence.

In this experiment, nearly genetically identical glochidia were used to infest naive brown trout hosts. Therefore, host age could be identified as an important interaction factor. However, in their natural environment, hosts are most likely to be infested by a wider range of parasitic species, and the scenario could be very different. In these circumstances, age-dependent host resistance against one parasite could reduce the immune defence against another one (Rohr et al., 2010). Thus, the cost of resistance is important to consider when examining the ecology and evolution of host-parasite interactions (Rigby et al., 2002).

5.2.3 Virulence

The results in Chapter 4 reveal a difference in glochidial virulence (host mortality) among two salmonid host species, with higher mortalities observed in the infested (less suitable) brown trout hosts, compared to the principal salmon host (Chapter 4). However, virulence is not the property of the parasite alone, but is regarded as the outcome of host-parasite interactions (Read, 1994). It is governed by the interactions

between the host and parasite genotypes, and environmental factors (Ewald, 1983; Mackinnon et al., 2002; Day & Burns, 2003; Perlman & Jaenike, 2003; Lambrechts et al., 2006; Salvaudon et al., 2007). Several examples have shown that virulence differs among different host-parasite genotype x genotype combinations (Lambrechts et al., 2006; Salvaudon et al., 2007; Bose et al., 2016). The differential virulence observed in Chapter 4 demonstrates how this occurs in the *M. margaritifera* host-parasite interaction. In the context of conservation, this could be an important factor to consider when choosing suitable hosts.

A host is often infested with parasites of multiple genotypes (of the same species). In these circumstances, virulence will be governed by the interaction between the host genotypes and all the parasitic genotypes, and this often leads to an increase in virulence (Riguad et al., 2010; Alizon et al., 2013; Bose et al., 2016). There are several different ways in which interaction between coinfecting parasites can influence virulence. For example, a host is a limited resource and the presence of two or more parasitic genotypes could lead to competition for resources, resulting in higher virulence (Taylor et al., 2005; Lagrue et al., 2011; Råberg, 2014; Klemme & Karvonen, 2019). Moreover, infecting parasitic genotypes of the same species most likely vary in their degree of virulence. When a host is infected with parasitic genotypes that have different degrees of virulence, the more virulent genotype would grow faster. This would result in higher virulence, and eventually host death (Bose et al., 2016). In addition, some studies have shown that genotype x genotype interactions between coinfecting parasites lead to an increase or a decrease in virulence (Bose & Schulte, 2014). In Chapter 4, the fish in the experiment were infected with multiple glochidial genotypes, and this probably also contributed to the observed variation in virulence.

In rivers, wild salmonid hosts may not only be infested with multiple genotypes of the same parasite, but possibly also by several different species of parasites. Some studies have shown that the interaction between coinfecting parasitic species also has an influence on the degree of virulence (Seppälä et al., 2009; Louhi et al., 2015). For example, Louhi et al. (2015) examined the interaction between bacterial strains

(*Flavobacterium columnare*) and trematode (*Diplostomum pseudospathaceum*) genotypes when they infect rainbow trout. They observed that host mortalities were higher in the presence of multiple infestations. Under natural conditions, the interactions between glochidia and other coinfecting parasitic species may influence the outcome of the infection, for example an increase or decrease in virulence, and this could influence the evolutionary dynamics between the glochidia and their hosts (Seppälä et al., 2009; Louhi et al., 2015). It is therefore usually difficult to predict the degree of glochidial virulence under natural conditions, due to the presence of other parasitic species. In Chapter 4, naive farm reared hosts were used. Therefore, it is less likely that an interaction between coinfecting parasitic species could have had an influence on the results.

Water temperature is an important abiotic factor that has been reported to modify host-parasite interactions (Lazzaro & Little, 2009; Studer et al., 2010; Scharsack et al., 2016). For example, temperature not only has an influence on the host immune response, resistance and survival, but also on the metabolic rates, infectivity and virulence traits of a parasite (Lazzaro & Little, 2009; Studer et al., 2010; Scharsack et al., 2016). In the *M. margaritifera* host-parasite system, both glochidial development and the salmonid host immune response are temperature dependent (Jansen et al., 2001). When water temperatures are low, the salmonid immune response is suppressed (Hruška, 1992; Hochwald, 1997; Alcorn et al., 2002; Ieshko et al., 2016), and these conditions are believed to enhance glochidial encystment and metamorphosis (Roberts & Barnhart, 1999; Taeubert et al., 2014; Ieshko et al., 2016). This could be important, because a strong immune response may also contribute to the degree of parasitic virulence. Thus, temperature variation may have an influence on the glochidial infestation window, because the relation between host resistance and parasite infectivity may differ at different temperatures (Lazzaro & Little, 2009; Scharsack et al., 2016).

Other factors, such as species-dependent differences in immune response, environmental conditions (other than temperature), and glochidial load, could also contribute to the host-parasite genotype x genotype interactions under natural

conditions (Bose et al., 2016; Mahmud et al., 2017). The immunogenetic diversity of the host major histocompatibility complex (MHC) is another factor that influences host-parasite compatibility. Studies have shown that specific MHC alleles in *S. salar* are associated with resistance to *Aeromonas salmonicida* (Langefors et al., 2001; Grimholt et al., 2003; Kjøglum et al., 2008) and myxozoa (Dionne et al., 2009). Resistance in certain brown trout strains to *Lactococcus garvieae* and *Yersinia ruckeri* are associated with an increased expression of MHC class I genes (Ozturk. et al., 2019).

Previous studies have shown that high glochidial loads result in host mortalities, compared to low to moderate loads (Treasurer et al., 2006; Taeubert & Geist, 2013). Unfortunately, it was not possible to examine the glochidial load on the infested dead hosts in Chapter 4. This could have shown if the glochidal load was a contributing factor towards host mortality.

M. margaritifera is an example of a non-trophically transmitted parasite that requires a single host in its life cycle in order to metamorphose, and also for down-stream migration. The glochidia require 9–11 months in order to metamorphose. Host survival for this entire duration is therefore essential for their survival. The virulence trade-off hypothesis predicts that optimal virulence is related to the transmission of the parasite (Anderson & May, 1982; Alizon et al., 2009), therefore one would expect *M. margaritifera* glochidia to have low virulence on the most suitable hosts. The lower glochidial virulence on the principal host may not necessarily indicate 'winnerless coevolution', but could be due to parasite specialisation on the host species that it is most likely to encounter. Nevertheless, specialisation in *M. margaritifera* could be related to the ancestral distribution of pearl mussel populations and the salmonid host, and a possible coevolution between them.

Overall, the results of all the experiments indicate that the phenotype of a host-parasite interaction is influenced by a complex interplay between host-parasite genotypes, as well as host-, parasite- and environmental factors. Although several

important factors were identified in this study, further research is required to improve our knowledge of host-parasite interactions in *M. margaritifera.*

5.3 Parasitic nature of *M. margaritifera* glochidia

In line with previous studies (Karna & Milleman, 1978; Bauer, 1987; Taeubert & Geist, 2013; Chowdhury et al., 2019), the results of this study also revealed the clear parasitic nature of *M. margaritifera* glochidia. Elevated haematocrit values were observed in hosts infested with glochidial loads exceeding 200 glochidia per fish (Chapter 3). It has been believed that low to moderate glochidial loads do not have a significant detrimental effect on hosts, although very high glochidial loads can lead to host mortality (Treasurer et al., 2006; Taeubert & Geist, 2013). Although the test fish (Chapter 3) had glochidial loads that were well within the recommended range of 5–100 glochidia per gram fish (Taeubert & Geist, 2013), they displayed respiratory stress, as indicated by their elevated haematocrit values. In addition, glochidial load was found to have a negative influence on host condition (Fulton's condition factor). The lower condition factor in 0+ hosts could be interpreted as a result of higher glochidial virulence in these hosts, or a cost of resistance.

Several studies have shown that parasites can manipulate host behaviour, for example make them more susceptible to predation or change their habitat preference (Lagrue et al., 2007; Mikheev et al., 2010; Lafferty & Shaw, 2013; Horký et al., 2014). Lafferty and Shaw (2013) proposed that less sophisticated parasites can manipulate their hosts by energetic drain. Denic et al. (2015) used stable isotope signatures to show nutrient flow from the salmonid host to the glochidia. A host which is starved for nutrients or has a lower condition could show a decrease in activity (Lafferty & Shaw, 2013). Salmonid hosts infested with *M. margaritifera* glochidia have been observed to show reduced drift foraging success (Österling et al., 2014), reduced host dispersal (Horký et al., 2014) and reduced swimming performance (Taeubert & Geist, 2013). Moreover, Filipsson et al. (2018) observed that high glochidial loads lead to the host being less active, capturing less prey and displaying more subordinate behaviour, compared to those that were less infested. These results are believed to be

related to a poor energetic status of the host and respiratory stress. Horký et al. (2019) observed that infested brown trout preferred habitats with different thermal regimes compared to uninfested hosts. They proposed that this was related to the optimal temperature preference of glochidia. Although the behaviour of infested hosts was not examined in this study, it is reasonable to assume that the lowered condition of the 0+ hosts and elevated haematocrit values in heavily infested hosts could have had an influence on host behaviour, i.e. causing a decrease in their activity or feeding.

In Chapter 4, a higher glochidial virulence (measured as mortality) was observed on the less suitable salmonid host species. To the best of my knowledge, I believe that this is the first study to show differential glochidial virulence among two salmonid host species, and where host mortality is associated with host suitability. Furthermore, and as an indirect effect, differential parasitic virulence can change the competitive relationship between hosts, and hence act as a factor influencing the regulation of host densities (Price et al., 1988; Thomas et al., 1995). The differential virulence observed suggests that brown trout, whose fitness was most affected by glochidial virulence, would be at a selective disadvantage compared to the less affected salmon host, especially in areas of dense salmon mussel populations (Price et al., 1988; Schall, 1992; Thomas et al., 1995; Lefèvre et al., 2008). Over time, this could affect trout density and maintain the frequency of salmon hosts. Overall, these results (Chapters 3 and 4) clearly show that glochidial infestation affects the salmonid host in terms of reduced condition, respiratory stress and even mortality. Although the hosts do benefit from the positive influence of freshwater pearl mussels in their ecosystem, the negative effects of parasitic glochidia could possibly outweigh these benefits.

The relationship between *M. margaritifera* and their salmonid hosts has been previously classified as either i) symbiosis-protocooperation because of their positive influence in aquatic ecosystems (described in Chapter 1) (Ziuganov et al., 1994; Skinner et al., 2003; Geist, 2010), or ii) phoresy because glochidial larvae are believed to benefit from their upstream dispersal along with their hosts (Barnhart et al., 2008). However, several authors have classified the relationship between *M.*

margaritifera and their salmonid hosts as parasitic (Karna & Milleman, 1978; Bauer, 1987; Taeubert & Geist, 2013; Chowdhury et al., 2019) because i) salmonid hosts mount an immune response when infested and large numbers of glochidia are lost 7 days post infestation (Bauer, 1987; Hastie & Young, 2003a), and ii) naive hosts develop acquired immunity against future glochidial infestation (Bauer, 1987; Bauer & Vogel, 1987; Chowdhury et al., 2018).

The results of this study also give evidence of the parasitic nature of *M. margaritifera*. In line with Filipsson et al. (2017), glochidial infestation was found to result in elevated haematocrit values (Chapter 3). Also, 0+ hosts displayed a lowered condition (Chapter 3), and more importantly, infestation caused host mortalities (Chapter 4). Spleen enlargement, respiratory stress, impaired swimming and impaired growth have also been reported in other studies (Taeubert & Geist, 2013; Thomas et al., 2014; Chowdhury et al., 2019).

5.4 Genetic diversity and selection on host gills

The results in Chapter 4 reveal that glochidia from individual mothers, from a population with salmon as the principal host, were able to infest both the salmonid host species. The higher genetic diversity associated with salmon-mussel populations explains the ability of mothers to infest the less suitable brown trout hosts (Karlsson et al., 2014; Geist et al., 2018). Although the brown trout hosts had higher mortality, several thousand juvenile mussels excysted from those that survived. This shows that when suitable hosts are scarce, for example due to changes in the environment, mussel populations have the potential to use a less optimal host as well, to ensure survival.

Freshwater pearl mussels are sperm casters, and a single mother can be fertilised by up to 15–20 males in a single mating event (Young & Williams, 1984b; Wacker et al., 2018). Multiple paternity can result in high genetic diversity among the offspring within a population, and also reduce the likelihood of inbreeding (Leslie & Vrijenhoek, 1977; Robbins et al., 1987; Moran & Garcia-Vazquez, 1998; Mäkinen et

al., 2007; Bai et al., 2011; Wacker et al., 2018, 2019b). A higher genetic diversity will allow glochidia to infest a wider range of salmonid hosts (Carius et al., 2001; Schmid-Hempel, 2001; Lambrechts et al., 2005; Little et al., 2006; Barribeau et al., 2014; Wacker et al., 2018). Individual and population level genetic diversity is associated with fitness and the ability to adapt to a changing environment, which will ensure the long-term survival of a species (Reed & Frankham, 2003; Markert et al., 2010).

Karlsson et al. (2014) and Geist et al. (2018) examined the genetic structure of pearl mussel populations in Norway and Ireland respectively, and observed that this was associated with their host use. They observed that salmon-mussels displayed higher genetic diversity within populations, and lower genetic differentiation between populations, compared to trout-mussels, which displayed lower genetic diversity within populations, but greater differentiation between populations. Geist et al. (2018) suggested that these patterns in population genetic structure could be explained by the differences in host use, geographic isolation, differences in habitat and population demographic effects. Karlsson et al. (2014) observed that pearl mussel populations that primarily infest Atlantic salmon are usually found in rivers where both salmon and brown trout coexist naturally. They also observed that trout-mussel populations were usually found in parts of the rivers where migration of fish was blocked by natural barriers (landlocked trout). Therefore, a lower geneflow and stronger genetic differentiation is expected in trout-mussel populations (Wacker et al., 2019a). In Chapter 4, the higher genetic diversity associated with salmon-mussel populations most likely facilitated the infestation of few trout hosts. However, it is not known whether the narrow genetic diversity of trout-mussel populations allow them to infest salmon hosts. Genetic studies are essential in order to assess the genetic diversity of *M. margaritifera* populations, and they are a necessary tool for their management, conservation and breeding strategies (Taeubert & Geist, 2017; Geist et al., 2018). As glochidial encystment, growth and development are tightly coupled with their salmonid host, the salmonid host (individuals, species or strains) will be a filter for the success of different glochidial genotypes. When new generations of *M. margaritifera* are produced in captive breeding programmes, it is

especially important to ensure that their genetic diversity is maintained. This can be achieved by using hosts of high genetic diversity.

5.5 Conservation implications

Evidence from this study has shown that glochidial infestation success, post parasitic fitness and virulence are associated with host suitability. These results could be used to improve conservation strategies, such as artificial infestation of fish for captive breeding or release into the wild. Moreover, the results also give an indication of how future environmental changes (warmer climate) could lead to a further decline in pearl mussel populations and their salmonid hosts. Different selection of glochidia on the hosts could lead to changes in the diversity of pearl mussel populations.

5.5.1 Captive breeding

Based on the results in Chapter 3, it is recommended that 1+ naive hosts are preferably used in captive breeding programmes. This will minimise the possible selection and genetic drift effects, and also maximise the production of young mussels. From an ethical point of view, obtaining the largest number of juvenile mussels from the most suitable 1+ hosts will reduce the number of fish required for artificial infestation.

In this experiment, the 1+ hosts displayed a high tolerance to glochidial infestation which was mirrored by their high host condition (Fulton's condition factor). In comparison, the 0+ hosts were resistant to glochidial infestation, and this resulted in a lower host condition. In addition, the juvenile mussel size was larger on hosts with the highest host condition. Österling and Larsen (2013) also observed a positive relationship between larval growth and host condition. The high tolerance to infestation (i.e. weaker immune response and thus lower cost of resistance) displayed by the 1+ hosts probably provided the glochidia with conditions beneficial for their growth and development. Lysne et al. (2006) observed that male *Gadus morhua* hosts infested with the copepod parasite *Lernaeocera branchialis* displayed a higher growth compared to uninfested hosts. The authors proposed that uninfested hosts had

paid an energetic cost for being resistant, and therefore had decreased growth. Therefore, the use of tolerant hosts will ensure good glochidial encystment and growth, as well as the well-being of the fish.

In Chapter 4, glochidial infestation resulted in host species dependent virulence, and higher mortalities were observed in the less suitable hosts. In Chapter 3, glochidial loads exceeding 200 glochidia per fish resulted in respiratory stress, as displayed by the elevated haematocrit values. Consequently, it is recommended that artificial infestation programmes should ensure low infestation rates on suitable hosts, as this can ensure good host condition and survival of infested fish used for captive breeding or release into streams. Good host condition promotes the successful release of juvenile mussels and their natural dispersal when infested fish are released in a river habitat (Jones et al., 2006; Thomas et al., 2010; Taeubert & Geist, 2013; Filipsson et al., 2017). Because glochidial growth, development and survival are dependent on host survival and condition, conservation efforts should focus on methods that can guarantee this (Taeubert & Geist, 2013; Filipsson et al., 2017). This can also help to preserve the genetic diversity of mussels, because the hosts are an important filter.

The lack of juvenile mussel recruitment has been associated with poor post parasitic survival (Buddensiek et al., 1993). Therefore, in most captive breeding programmes, juvenile mussels are collected and then maintained in plastic boxes, or in artificial rivers, until they are ready for release into their natural habitat (Thomas et al., 2010; Gum et al., 2011; Eybe et al., 2013). In Chapter 2, juvenile mussels that had the longest parasitic phase also had a size, growth and survival advantage. It would be most effective to use these late excysters for rearing in boxes or artificial rivers. However, there could be a risk associated with artificial selection (discussed below). Generally, juvenile mussels that excyst early are small and usually have a very poor survival in boxes (Eybe et al., 2014; personal observation, 2012–2019). In Norway, juvenile mussels (1–3 years old) have been released in plastic mesh boxes (Hruška boxes, further developed by Michael Lange) with clean sediment. In such boxes, or under pure natural conditions, mussels with the longest parasitic phase will have better resources (larger size and better growth rate) to start their benthic

developmental phase, and therefore to survive their first winter. However, since juvenile mussels have a very specific habitat requirement, the quality of the stream/river sediment should be assessed before releasing the captive bred juveniles.

5.5.2 Maintaining genetic diversity

Captive breeding of juvenile mussels is the most widely used method in the conservation of *M. margaritifera* (Thomas et al., 2010; Gum et al., 2011). An important concern with the method is that it could alter the genetic composition of a species (Hoftyzer et al., 2008). For example, Wilson et al. (2012) observed a small but significant level of genetic differentiation between captive bred mussels and their parent mussels, which suggested a possible founder effect. In addition, Kyle et al. (2016) also observed significant levels of inbreeding in captive bred juvenile mussels, despite the rotation of broodstock mussels. Loss of genetic diversity could negatively influence mussel fitness and their evolutionary adaptive potential (Reed & Frankham, 2003; Jones et al., 2006; Hoftyzer et al., 2008; Geist, 2010; Markert et al., 2010; Wilson et al., 2012; Donaldson et al., 2019).

In Chapter 4 it is shown that glochidia from individual mothers were able to infest and excyst from both the principal and less suitable hosts. Broodstock mussels, especially when dealing with small and isolated populations, should be carefully selected in order to ensure that no rare alleles are lost. In *M. margaritifera* populations with low densities, females can switch to hermaphroditism (Bauer, 1987). In such circumstances, low contribution from males can result in detrimental genetic consequences (Cauwelier et al., 2009). Using only a small number of founder mussels could lead to loss of genetic diversity or inbreeding depression (Jones et al., 2006; Hoftyzer et al., 2008). However, there are currently no studies that recommend the minimum number of individuals that should be used to avoid this (Wilson et al., 2012). Also, with the infestation and developmental success of glochidia tightly coupled with their hosts, careful consideration should be employed when choosing suitable hosts for artificial infestation. A lack of genetic diversity in the host fish population could for example contribute to the undesired loss of genetic diversity in the mussel population (Stoeckle et al., 2016; Boon et al., 2019). Captive breeding of

M. margaritifera involves artificially selecting the fittest juvenile mussels, rearing them in boxes and eventually releasing them into rivers (Jones et al., 2006; Donaldson et al., 2019). This activity can in itself potentially cause a loss of genetic diversity and heterozygosity of captive bred mussels, which in turn can influence survival in the natural environment (Frankham et al., 2002). However, Sten Karlsson (personal communication) did not observe any genetic differences between captive bred juvenile mussels reared at Austevoll that excysted early and late during the excystment period. Captive breeding methods should aim to ensure that a high level of the genetic diversity of a population is retained, and genetic diversity analyses of populations is the tool that can help to ensure this (Soulé et al., 1986; Geist, 2010; Wilson et al., 2012). Conservation efforts should therefore rely on genetic studies, as well as ecological studies, in order to achieve the best possible results (Geist, 2010).

5.5.3 Temperature

Being poikilothermic, the growth and reproduction of *M. margaritifera* is dependent on water temperatures (Hastie & Young, 2003a; Skinner et al., 2003; McIvor & Aldridge, 2008; Österling et al., 2008; Taeubert et al., 2013). In Chapter 2, temperature was identified as an important factor governing excystment, with higher temperatures decreasing the duration of the parasitic phase. Variation in temperature can delay reproduction within rivers by several weeks during cold years (Hastie & Young, 2003a; Hastie et al., 2003), and it can influence glochidial metamorphosis (Hruška, 1992; McIvor & Aldridge, 2008), growth (Larsen, 2005), survival (Jansen et al., 2001), duration of the parasitic phase and release of glochidia from their cysts (Lefevre & Curtis, 1912; Hruška, 1992; Ziuganov et al., 1994; Larsen, 2005; McIvor & Aldridge, 2008; Eybe et al., 2014).

Glochidial encystment and development is also dependent on the host immune response (Nezlin et al., 1994). Low water temperatures lead to a suppression of the salmonid immune response (Hruška, 1992; Hochwald, 1997; Alcorn et al., 2002; Ieshko et al., 2016), and this is believed to enhance glochidial encystment and metamorphosis (Roberts & Barnhart, 1999; Taeubert et al., 2014; Ieshko et al., 2016). Glochidial release typically begins in autumn when water temperatures are low

(Ziuganov et al., 1994; Larsen, 2005), and some authors speculate that pearl mussels have evolved to release glochidia at this time in order to take advantage of the suppression of the host immune response (Roberts & Barnhart, 1999; Taeubert et al., 2014; Ieshko et al., 2016). Therefore, in artificial breeding programmes, knowledge of the optimal temperature requirements for *M. margaritifera* will help in maintaining effective temperature regimes for successful glochidial encystment (Hastie & Young, 2003a). Taeubert et al. (2013) recommended that infested hosts should be maintained at temperatures between 10–12 °C for the duration of the glochidial parasitic phase. Once the glochidia have reached their optimal size, temperatures should be increased to begin excystment. In Chapter 2, the water temperature in the fish holding system followed the natural temperature variation of the source lake, and was between 5.7 and 17.0 °C. Although juvenile mussel excystment started at 11 °C, the maximum number of juveniles excysted between 13–17 °C. This result is in line with Taeubert et al. (2013), where higher temperatures are required for the excystment of juveniles.

5.6 The Red Queen and *M. margaritifera*

The results of this study have shown that host-parasite interactions between *M. margaritifera* and their salmonid hosts have an influence on both the host and the parasite. Such interactions can influence the evolutionary trajectories of both the host and the parasite (Chong & Roe, 2018). According to the Red Queen hypothesis, host and parasite are engaged in an oscillatory dynamic that affects the abundance of interacting genotypes. In the pearl mussel salmonid host coevolutionary relationship, it can be difficult to observe the Red Queen oscillations (to pinpoint where the cycle has reached) in experiments, because of the longer generation time, and life span, of the parasite in this host-parasite model. In this relationship, the parasite is expected to experience a stronger selection pressure on compatible host genotypes, because its survival depends on host compatibility (Douda et al., 2017). Studies have shown that freshwater pearl mussel populations display high variability in host-specificity, yet no study has given clear evidence of local adaptation of *M. margaritifera* to the sympatric salmonid host (Taeubert et al., 2010; Österling & Larsen, 2013; Salonen et

al., 2017; Taeubert & Geist, 2017). Thus, it may be that pearl mussels have an adaptation against the non-specific immune response of the host fish, and they are able to exploit the host species they are most likely to encounter (Douda et al., 2017). In comparison, the hosts are expected to experience a weaker selection for resistance to glochidia (Douda et al., 2017). This is because the parasite is distributed across a smaller area of the host's total distribution range, and it infests only the freshwater (young) stage of the host (Douda et al., 2017). However, glochidial infestation has a negative effect on their hosts, which is evident from this study, as well as several others (Taeubert & Geist, 2013; Horký et al., 2014; Douda et al., 2017; Filipsson et al., 2017; Chowdhury et al., 2019). The resulting cost of infestation to the host suggests that pearl mussel glochidia are indeed a selective force, and this can result in potential mussel-salmonid host coevolution (Douda et al., 2017; Chowdhury et al., 2019).

Most studies, including this one, examine the host-parasite interactions between a single host-parasite pair. However, hosts and their parasites exist in diverse multispecies communities, where interactions between all the coinfecting species may influence coevolution (Karvonen et al., 2009; Betts et al., 2016). Generally, *M. margaritifera* depend on the presence of naive hosts for encystment, because hosts exposed to glochidia are reported to develop acquired immunity against future infestations (Bauer & Vogel, 1987; Rogers-Lowery et al., 2007; Chowdhury et al., 2018). In nature, the probability of finding uninfested salmonid hosts is very low, and most hosts would be exposed to a wide array of parasites, including *M. margaritifera* glochidia. Under such circumstances, a host should resist local parasites and pathogens in order to optimise for its own survival and reproduction (Eizaguirre et al., 2012a). The major histocompatibility complex (MHC) is a genetic defence mechanism that has developed in vertebrates in response to evolutionary pressure from parasites and pathogens (Eizaguirre & Lenz, 2010). Eizaguirre et al. (2012a) observed local adaptation in the MHC genotypes of lake and river populations of three spined sticklebacks (*Gasterosteus aculeatus*) to population-specific (lake or river) parasites. The close association between MHC alleles and a parasite leads to Red Queen dynamics, and observations that match these oscillatory dynamics have

been made for *G. aculeatus* and *Gyrodactylus* species (Eizaguirre & Lenz, 2010; Eizaguirre et al., 2012b). Eizaguirre et al. (2012b) proposed that the frequency shifting of adaptive MHC alleles could decrease the prevalence of specific parasites, and also "open the door" for other parasites.

A host can become infected with several parasitic species at the same time, or during a sequential infection (Vaumourin et al., 2015). The interaction between all coinfecting parasitic species may shape the structure of the parasitic community within a host (Poulin, 2001). Their presence may facilitate or hinder (directly or indirectly) subsequent infections by other parasites, such as *M. margaritifera* glochidia (Vaumourin et al., 2015; Gopko et al., 2017). For example, mechanical damage or suppression of the host immune response can facilitate infection by another parasitic species (Combes, 2000; Pedersen & Fenton, 2007). Gopko et al. (2017) observed that pre-infection with *M. margaritifera* glochidia made *S. trutta* more susceptible to infestation by *Diplostomum pseudospathaceum*. Ziuganov (2005) also observed that glochidial infestation leads to an increase in non-specific resistance to the fungal pathogen *Saprolegnia*. Exclusion or on-going competitive interaction between coinfecting parasites may result from an activation of the host immune system, or from competition for resources as a result of immune activation, or from direct interactions between coinfecting parasitic species (Poulin, 2001; Rigaud et al., 2010). Furthermore, pre-infection by one parasitic species can result in resistance to another parasitic species in the host (cross-resistance) (Buchmann et al., 1999; Larsen et al., 2002). For example, Chowdhury (2018) observed that pre-infection with *M. margaritifera* glochidia reduced the susceptibility of *S. trutta* to the bacterial disease caused by *Flavobacterium columnare*. Hosts infested with glochidia had a higher survival compared to the control fish, and the author proposed that the mechanism for protection could be related to the enhancement of non-specific immunity or changes in the gill structure. Moreover, Dodd et al. (2005) observed that glochidia transformation success of *Lampsilis reeveiana*, *L. abrupta*, *Villosa iris* and *Utterbackia imbecillis* was lower on largemouth bass hosts (*Micropterus salmoides*) that were previously infested with *L. reeveiana*. In contrast, Chowdhury et al. (2018)

observed that pre-infection with the duck mussel *Anodonta anatina* did not result in cross immunity against infestation with *M. margaritifera* glochidia in *S. trutta*. All the experiments in this study were performed under common garden conditions, using a single host-parasite pair. It is difficult to predict how the presence of other coinfecting parasites could influence the phenotype of the glochidia-host interaction. However, it is possible that coinfecting parasites could play a role in the glochidia-host evolutionary trajectories.

When a parasite has a lower detrimental effect on host fitness, it may lead to a weaker selection for immunogenetic counter-adaptations in the host. *M. margaritifera* is a short-term parasite that does not reproduce in its host. Typically, most salmonid hosts become infested with glochidia only once in their lifetime. In nature, however, they may experience repeated exposure to other, more virulent, parasites that cause significantly more damage. Thus, one may speculate that the salmonid hosts will experience stronger selection for counter-adaptations to these parasites, compared to *M. margaritifera*.

In a host-parasite system where the parasite has a very long life span, it is difficult to observe the oscillatory dynamics of the Red Queen hypothesis. In *Through the Looking-Glass* (1871), Alice is advised by the Rose to walk the opposite way in order to approach and talk to the Red Queen. Lythgoe and Read (1998) suggested that one could adopt this advice from the Rose: Look back in time and compare the infection frequencies of host genotypes that were formerly common with host genotypes that were formerly rare. This may help to shed light on the *M. margaritifera* salmonid host-parasite coevolutionary relationship.

5.7 Outlook

The specific habitat requirement of juvenile mussels makes this species particularly vulnerable to environmental change (Lydeard et al., 2004; Bogan, 2008; Geist, 2010). Although captive breeding is important to ensure the survival of the species, improving the natural habitat is also essential. Several rivers in Norway still face the

threat of eutrophication, siltation and acidification. These conditions must be improved to ensure the survival and growth of captive bred juvenile mussels that are released back into their natural habitat. Until the situation in rivers can be improved, however, a large part of conservation will continue to depend on captive breeding.

In a BBC interview (2001), Sir David Attenborough stated: "*The only way to save a rhinoceros is to save the environment in which it lives, because there's a mutual dependency between it and millions of other species of both animals and plants. And it is that range of biodiversity that we must care for – the whole thing – rather than just one or two stars*." Future conservation and management strategies should therefore not only focus on conserving the freshwater pearl mussel, but also on protecting and preserving the freshwater ecosystem in its entirety.

Based on observations made during this study, here are some suggestions for future work:

1. Juvenile mussels that had a longer parasitic phase had a fitness benefit in terms of size, survival and growth rate, compared to those with a shorter one. An examination of the lipid and polysaccharide content among juvenile mussels with different parasitic phase lengths will give information about the energy reserves with which the juveniles begin their life as free living organisms. Moreover, it could also explain the difference in growth rates that was observed post excystment under equal temperature conditions. The morphological development of these mussels could also be examined to see if the mussels that excyst early are developmentally premature, compared to those that excyst later.
2. The results of this study have shown that glochidial infestation success, post parasitic fitness and even virulence is associated with host compatibility. Future studies should further examine the factors that influence host-parasite compatibility, such as the immunogenetic diversity of the major histocompatibility complex (MHC). Specific MHC alleles in *S. salar* are associated with resistance to *Aeromonas salmonicida* (Langefors et al., 2001; Grimholt et al., 2003; Kjøglum et al., 2008) and myxozoa (Dionne et al., 2009).

Resistance in certain brown trout strains to *Lactococcus garvieae* and *Yersinia ruckeri* are associated with an increased expression of MHC class I genes (Ozturk. et al., 2019). For example, one could examine if individual MHC heterozygosity is related to glochidial load, or if specific MHC alleles increase host resistance or tolerance to glochidial infestation.

3. In Chapter 4, differential virulence was observed among the two salmonid host species. However, the underlying cause of the higher mortalities in the less suitable host species was not investigated. Further studies should investigate the glochidial densities, gill tissue histopathology, and haematology of infested dead hosts, to examine the host dependent response to glochidiosis and the possible factors that led to their mortality. Similar to the previous suggestion, the immunogenetic diversity of the MHC could also be examined in the salmon and trout hosts.
4. The presence of coinfecting parasitic species or cross-resistance due to pre-infection with another parasitic species could change the host-parasite compatibility in the wild. Thus, for example, glochidial virulence in the presence or absence of other parasites could be examined using the same experimental methods used in Chapter 4. This could help us understand if host-glochidial compatibility changes in the presence of other parasitic species.
5. The results presented in Chapter 4 showed that glochidia from individual mothers from a population with salmon as the principal host were able to infest both salmonid host species. This raises the question: are offspring from individual fathers or certain parent pairs able to infest either trout or salmon, or both the host species? It would be interesting to examine the parental contribution in influencing glochidial infestation success (abundance, growth) of the different salmonid host species. Individuals with higher individual heterozygosity are generally more successful in infesting any hosts, and studies could examine if there is a difference in the individual heterozygosity of juveniles that excyst from salmon and trout.
6. Trout-mussel populations are reported to have lower genetic diversity within populations, and higher genetic differentiation between populations (Karlsson et

al., 2014; Geist et al., 2018). Most of the trout-populations co-occur in rivers where only landlocked brown trout is present (Karlsson et al., 2014; Wacker et al., 2019a). Moreover, in rivers where salmon was introduced beyond its natural anadromous reach, no glochidia were found on salmon, only on resident trout (Larsen, 2006; Karlsson & Larsen, 2013). The hypotheses in Chapter 4 could be tested again, but this time with glochidia from a population that uses resident trout as its principal host. It would be interesting to see if such an experiment would reveal similar patterns again.

7. In Chapter 4, naive farmed (domesticated) *S. salar* and *S. trutta* were used as hosts for the *M. margaritifera* populations examined. However, these are not the local (coevolved) hosts for the pearl mussel populations used in the experiment. Therefore, further studies should test the hypotheses in Chapter 4, and examine if the patterns in infestation and virulence vary among farm reared and coevolved hosts from the natural habitat.
8. Temperature is an important variable that has an influence on the host and parasite fitness, and their interaction. Therefore, models to predict future temperature changes in river systems could also be helpful in planning conservation measures, especially when dealing with changes in the climate.

6. References

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