

# **Direct and indirect effects of invasive parasites on native blue mussels**

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*Mytilicola intestinalis* Steuer, 1902 affects its host  
*Mytilus edulis* Linnaeus, 1758 and modifies ecological interactions  
to other species in the Wadden Sea

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„Wenn Du fragst, was rechtes Wissen sei, so antworte ich, das, was zum Handeln befähigt.“

Hermann Ludwig Ferdinand von Helmholtz



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## Zusammenfassung

Mit der Zunahme biologischer Invasionen innerhalb des marinen Ökosystems wächst die Rolle von Parasiten in solchen Invasionsprozessen stetig. Wenn Parasiten selbst invasiv sind und auf heimische Arten überwechseln, können sie eine Reihe direkter und indirekter Effekte auf den neuen Wirt und die mit ihm interagierenden Arten ausüben. Direkte Effekte wirken auf vielfältige Weise auf den Wirt ein, daraus können wiederum indirekte Effekte hervorgehen. Indirekte Effekte können weiter auf die Interaktion zwischen Wirten und die mit ihnen wechselwirkenden Arten einwirken. Als Folge von beidem (direkten und indirekten Effekten) können Parasiten die Sensibilität eines Wirtes gegenüber weiteren Einflüssen, wie beispielsweise veränderten Klimabedingungen und/oder Sekundärinfektionen, beeinflussen. Dies hat tiefgreifende ökologische Auswirkungen für heimische Arten. Insgesamt bergen die Herausforderungen von Invasionsprozessen und dem Klimawandel das Risiko der Artenhomogenisierung und dem Aufkommen von Krankheiten. Vor diesem Hintergrund ist es eine der Hauptaufgaben der marinen Forschung zukünftige Einflüsse und Folgen von parasiten-beeinflussten Invasionen und dem Klimawandel abzuschätzen. In der vorliegenden Arbeit werden die direkten und indirekten Effekte des invasiven Parasiten *Mytilicola intestinalis* auf heimische Miesmuscheln untersucht. Die Arbeit gliedert sich in drei Kapitel, das erste Kapitel befasst sich mit den direkten Effekten, Kapitel zwei und drei mit den indirekten Effekten.

Um die Auswirkungen von *M. intestinalis* auf heimische Miesmuscheln zu untersuchen, habe ich verschiedene Experimente auf der Insel Sylt (Deutsche Bucht, Nordsee) durchgeführt. Zunächst habe ich Miesmuscheln künstlich mit dem Darmparasiten infiziert, um die späteren experimentellen Daten unter Hinzunahme bekannter Infektionsintensitäten zu interpretieren. In unterschiedlichen Labor- und Feldexperimenten habe ich verschiedene direkte Effekte des invasiven Parasiten auf seinen Wirt analysiert. Diese Untersuchungen beziehen Experimente zu Isotopenwerten von Stickstoff (von Muscheln, Parasiten und Nahrungsressourcen) und den Stoffwechselprofilen von parasitierten und nicht-parasitierten Muscheln sowie deren Wachstum und Kondition mit ein (Kapitel I). Die Ergebnisse zeigen, dass die stabilen Stickstoffisotopenwerte für den Parasiten im Vergleich zum Wirt selbst, erhöht sind. Es wird deutlich, dass der Parasit hauptsächlich Gewebe des Wirtes konsumiert. Die direkte Aufnahme von Wirtsgewebe resultierte in einem veränderten Stoffwechselprofil für infizierte Muscheln, wobei hauptsächlich Stoffwechselprodukte des Aminosäurezyklus beeinträchtigt waren. Unter Feldbedingungen wuchsen experimentell infizierte Muscheln langsamer als nicht-infizierte Muscheln. Unter Laborbedingungen zeigten parasitierte Muscheln eine geringere Kondition als nicht-parasitierte Muscheln auf. Dies deutet auf einen direkten negativen Effekt des Parasiten auf seinen Wirt hin. Darüber hinaus habe ich eine Langzeitstudie (von Oktober 2014 bis September 2015) über die monatliche Parasiteninfektion einer intertidalen Miesmuschelbank durchgeführt. Mittels der bekannten Parasitenintensität können die experimentellen Momentaufnahmen der direkten Effekte über die

Jahreszeiten hinweg extrapoliert werden. Der saisonale Verlauf der Parasitenpopulation zeigte, dass eine permanente aber variierende Belastung der Muscheln mit dem Parasiten besteht. Jeweils im Sommer und im Herbst wurde ein Reproduktionspeak des Parasiten verzeichnet (Kapitel I). Neben den direkten Effekten habe ich die indirekten Effekte des Parasiten untersucht. Dazu habe ich die Räuber-Beute-Beziehung infizierter und uninfizierter Miesmuscheln zu einem ihrer Hauptprädatoren, der Strandkrabbe *Carcinus maenas*, analysiert. Dafür habe ich drei Prädationsexperimente entworfen, die ich mit zwei unterschiedlichen Lebensstadien des Parasiten im Labor durchgeführt habe (Kapitel II). Im Ergebnis wurde deutlich, dass der invasive Parasit indirekt die Räuber-Beute Beziehung von Muscheln und Strandkrabben beeinflusste. Der Prädationsdruck auf Wirte, die mit juvenilen Stadien des Parasiten infiziert waren, war niedrig, wohingegen dieser hoch war, wenn Wirte mit adulten Parasitenstadien infiziert waren. Dieses sich umkehrende Muster wurde zu verschiedenen Zeiten im Jahr deutlich, (Kapitel II).

Schließlich habe ich krankheits-relevante Effekte des Parasiten auf die Immunantwort von Muscheln in einem Laborexperiment untersucht. Durch die Simulation verschiedener Infektionsszenarien habe ich den Krankheitsverlauf von parasitierten und nicht parasitierten Muscheln mit solchen verglichen, die zudem mit sekundären *Vibrio* spp. infiziert waren. Dazu habe ich die Phagozytoserate der Muschelhämozyten gemessen und die Vibriobelastung anhand der Kolonie bildenden Einheiten für die unterschiedlichen Infektionsansätze ermittelt. Dieses Experiment habe ich unter verschiedenen Temperaturkonditionen durchgeführt (Kapitel III). Das Infektionsexperiment zeigte, dass der invasive Parasit die Phagozytosekapazität von Muscheln beeinträchtigt, auch wenn dieser nicht direkt mit den Hämozyten der Muschel interagiert. Bei höheren Temperaturen führte dies zu einer erhöhten Anzahl Kolonie bildender Einheiten und dadurch zu einer erhöhten Mortalität von parasitierten Muscheln, die zusätzlich mit *Vibrio* spp. infiziert waren (Kapitel III). Indem ich die Ergebnisse verschiedener Labor- und Feldexperimente kombiniert habe konnte ich zeigen, dass der Parasit direkte und indirekte Effekte auf seinen Wirt ausübt. Eine Infektion mit dem Parasiten limitiert die verfügbaren Ressourcen für das Wachstum (direkte Effekte) aber auch für die Immunantwort von Miesmuscheln gegen sekundäre Infektionen (indirekte Effekte). Dies zeigt, dass direkte Effekte des Parasiten wiederum die Wechselwirkungen seines Wirtes zu dritten Arten verändern können. Die vom Lebensstadium des Parasiten abhängige Manipulation des Prädatorenverhaltens repräsentiert einen extremen Fall von solchen indirekt veränderten Interaktionen. Dies verdeutlicht, dass indirekte Effekte das Ausmaß von direkten Effekten übertreffen können. Um die ökologischen Auswirkungen von biologischen Invasionen im Allgemeinen und die von invasiven Parasiten im Speziellen abschätzen zu können, müssen indirekte Effekte und deren Wechselwirkung mit der biotischen und abiotischen Umwelt mit in Betracht gezogen werden.



## Summary

The role of parasites in invasion processes have become an increasing phenomenon due to the continuous increase in the rate of biological invasions in marine ecosystems. When parasites themselves are invasive and spill over to native species, they can exert a number of direct and indirect effects on their new host and interacting species. Direct effects often affect the new host in multiple ways that result in further indirect effects downstream. Moreover, the occurrence of indirect effects might change the interaction of the host to interacting species. As a result of both, direct and indirect effects, parasites can change the sensitivity of the host against further impacts like climate change and/or secondary infections. This can have profound ecological consequences for native biota. In combination, the challenges of invasion processes and climate change bear the risk of species homogenization and disease emergence. Against this background, it is an urgent task of marine science to assess future impacts and consequences of parasite invasions and climate change. The present study investigates the direct and indirect effects of the invasive parasite *Mytilicola intestinalis* on native blue mussels. The study is divided into three chapters; the first chapter addresses the direct effects, while the second and third chapter considers indirect effects.

To investigate the impacts of *M. intestinalis* on native blue mussels, I conducted different experiments on the island Sylt (German Bight, North Sea). Initially, I experimentally infected mussels with the intestinal parasite to interpret experimental data in the light of known infection intensities. Thereafter, I investigated several direct effects of the invasive parasite on its host, in both field and laboratory studies. Those investigations include analyses on the nitrogen isotope values of mussels, parasites and food sources, experiments on the metabolic profile of parasitized and non-parasitized mussels, as well as their growth and condition (Chapter I). The results showed an enrichment of stable nitrogen isotopes in the parasite compared to the host indicating that the parasite primarily consumes host tissue. This direct consumption of host tissue further resulted in an altered metabolic profile with most metabolites of the amino acid cycle being affected. Under field conditions, experimentally infected mussels grew slower than non-infected mussels. Under laboratory conditions, parasitized mussels showed a lower condition than non-parasitized mussels, indicating a direct negative impact of the parasite on its host. Furthermore, I executed a long-term survey (from October 2014 to September 2015) on the monthly parasite infestation at an intertidal mussel bed. With the known parasite infection intensity background in the field, the experimental snapshots could be extrapolated across seasons. The seasonal course of the parasite's population dynamics showed a distinct pattern with a permanent but varying burden of mussels by the parasite and the main parasite broods occurring in summer and autumn, (Chapter I). Next to these direct effects, I explored the indirect effects of the parasite. I looked at the predator-prey interaction of experimentally infected and uninfected blue mussels with one of their most prominent predators, the shore crab *Carcinus maenas*. To do so, I conducted three laboratory predation experiments

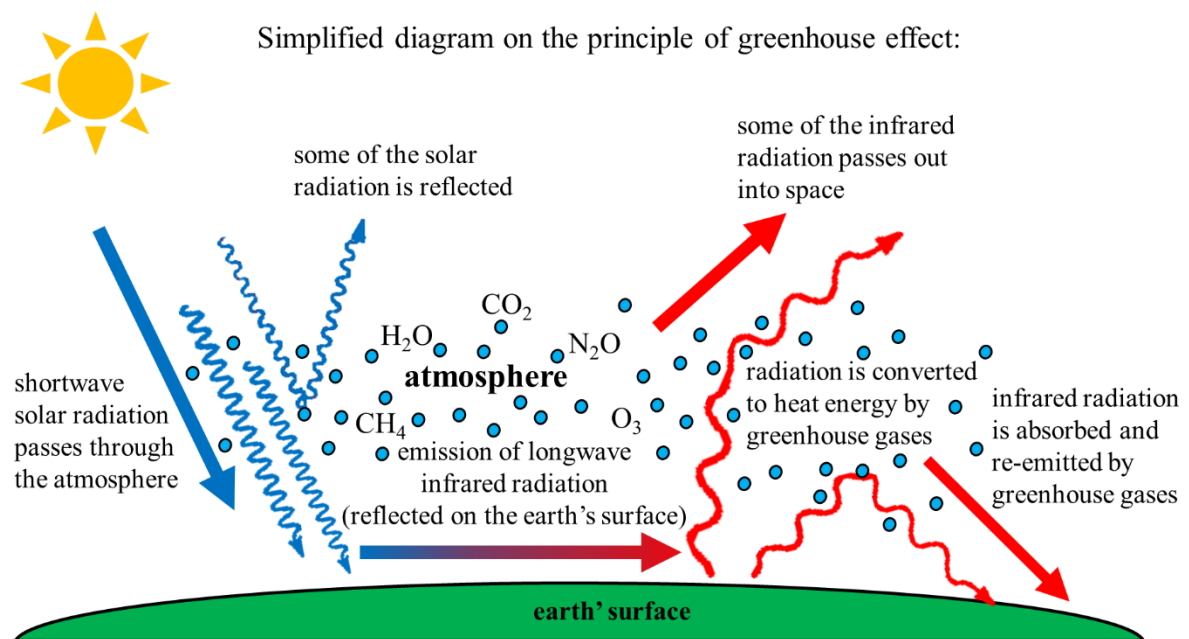
that I carried out with two different life stages of the parasite (Chapter II). The invasive parasite indirectly influenced the predator-prey interaction of mussels and shore crabs. Initially, predation pressure on hosts infected with juvenile parasite stages was low, whereas, this pattern was reversed when crabs were offered mussels infected with adult parasite stages at a different time of the year (Chapter II).

Finally, I investigated the trait-mediated effects of the parasite on the immune response of mussels to a secondary infection in a laboratory experiment. Simulating different infection scenarios, I compared the pathogenesis and immune response of parasitized and non-parasitized mussels to those that were additionally infected by secondary *Vibrio* spp. infections. For that purpose, I measured the phagocytosis rates of the mussel hemocytes and estimated the *Vibrio* load by evaluating the number of viable cells for the various infection treatments. This experiment was performed under different temperature conditions, (Chapter III). The infection experiment revealed that the invasive parasite limits the phagocytosis capacity of mussels without directly interacting with the hemocytes of the host. At higher temperatures, the decreased phagocytosis activity led to a higher *Vibrio* spp. load, resulting in an increased mortality of parasitized mussels when exposed to a secondary *Vibrio* spp. infection (Chapter III). Combining the results of laboratory and field experiments, I could show that an infection by the parasite *M. intestinalis* has various direct and indirect effects for the host. A parasite infection limits the resources available for growth (direct effects) but also for the immune response of mussels against secondary infections, (indirect effects). This demonstrates that direct effects of the parasite may indirectly modify the interaction of its host with third species. The life-stage dependent manipulation of predator behavior by the parasite represents an extreme case of such altered interactions and clearly shows that indirect effects can outweigh direct effects. Therefore, such indirect effects and their interactions with the biotic and abiotic environment need to be taken into account to fully assess the ecological consequences of biological invasions in general and parasitic invasions in particular.

## I. Introduction

### 1. Invasions in the German Wadden Sea & anthropogenic global change

The natural greenhouse effect is accelerated by anthropogenic processes like fossil fuel burnings and deforestation and leads to the emission of radiatively active gases in the atmosphere, Fig. I.1., (Burroughs, 2007). Thus, resulting in raised greenhouse effect that includes the increase of UV-light and global mean air temperature (Harley *et al.*, 2006). Hence, the world's oceans absorb more CO<sub>2</sub> and the expansion of water molecules raises the sea water levels (Pachauri *et al.*, 2014). This will result in a cascade of chemical and physical alterations for marine systems (Harley *et al.*, 2006).



**Figure I.1.:** The greenhouse effect. Modified after (Houghton *et al.*, 1995; Houghton *et al.*, 1990).

Besides global warming (Fig. I.1.) a rising number of environmental changes are attributable to human activities (Duarte, 2014; Steffen *et al.*, 2005). This includes globalization and ship traffic that increasingly introduces species with pathogens and parasites to new areas with ballast water tank discharge, ship fouling and aquaculture transfers representing the main introduction vectors for aliens, (Buschbaum *et al.*, 2016; Gollasch, 2006). Hence, compared to the open sea, marine coastal areas receive aliens more frequently (Cohen & Carlton, 1998; Grosholz, 2002). Additionally, the construction for coastal protection and offshore wind farms increases the amount of artificial hard substrates and thus, creates a basis of existence for introduced species (Buschbaum *et al.*, 2016). Anthropogenic formed introduction ways and artificial constructions together with rising water temperatures promote the establishment of aliens within new ranges (Buschbaum *et al.*, 2016).

The spread of biota to non-native areas produces species known as ‘alien species’ (Kowarik, 2010). Aliens are termed as ‘invasive’ when they evoke changes in the new range and threaten the indigenous biocoenosis (IUCN, 2000). They become established in a new area; when they bear more than three generations since their introduction or existence at least 25 years within the new area (Weigmann, 1996). Although the amount of species that are permanently introduced is high, the establishment of alien species within the new area is low according to the tens rule: 1 out of 10 wild species become introduced; 1 in 10 of those introduced species become established and 1 in 10 of those become a pest (Williamson & Fitter, 1996). The chance for species to be established depends crucially on finding a niche opportunity (Shea & Chesson, 2002; Stachowicz & Tilman, 2005). Geologically the Wadden Sea coast is a very young habitat, which can be characterized by its frugal benthic communities (Nehring, 2001). Since the re-colonialization of the North Sea after the last glacial period, the spectrum of species is markedly below its potential contingent (Reise *et al.*, 1999). The Wadden Sea ecosystem therefore offers a large number of potentially free ecological niches. Furthermore, the Wadden Sea ecosystem is highly dynamic. The alterations of the abiotic factors in the intertidal, permanently disturb species communities (Nehring, 2001). Therefore, foreign species do not hugely affect the stability of the native community of species but latently get incorporated into them (Nehring, 2001; Reise *et al.*, 1999).

Hence, at the German North Sea coast there exist no actual data from mussel habitats without containing neobiota and up to two new species establish annually (Buschbaum *et al.*, 2016; Büttger *et al.*, 2008; Nehring & Leuchs, 1999). Since the Discovery of America by Columbus in 1492, the number of aquatic alien taxa introduced into the German North Sea is more than 100 with species of Crustacea are one of the dominant taxa (Gollasch & Nehring, 2006; Lackschewitz *et al.*, 2015; Lackschewitz, 2017, personal communication). Currently, the increase of invasive species in the North Sea proceeds exponentially. Yet, species invasions within the European aquatic coasts did not lead to an extinction of native species or to a serious threat of the ecosystem (Reise *et al.*, 2006; Wolff, 2000). Thus, the net consequence is an expansion of the ecosystem functioning (Reise *et al.*, 2006). Nevertheless, negative effects of species invasions are to be expected and apply particularly to those cases where the invading species is a parasite.

## 2. Parasite-mediated direct and indirect effects of biological invasions

The impact of invasive species, proceeds via direct and indirect effects – the linkage between species interactions (Moon *et al.*, 2010). In general, the changes caused by invasive aliens can be positive for native species for example via habitat formation or amelioration of stress (Rodriguez, 2006; Thomsen, 2010). Invasive species can also have negative effects on native biota for instance by causing the decline of abundances of native species (Kideys, 2002). However, direct effects occur when species physically interact (Wootton, 1994). Those effects such as predation or competition are well described in invasion processes (Byers, 2009; López *et al.*, 2010; Rilov, 2009). Indirect effects on the other hand occur by altering habitats or food-web dynamics and can only arise when an intermediary species like a parasite is present (Baxter *et al.*, 2004; Wallentinus & Nyberg, 2007; Wootton, 1994). By modifying the density, the traits or the behavior of one species, the mediatory parasite can alter the interaction between the first species to other species (Dill *et al.*, 2003; Trussell *et al.*, 2006; Werner & Peacor, 2003). The outcome of ecological interactions and thus, the success of invasions can be determined via direct and indirect effects of parasites. Hence, parasites play a key-role in mediating invasion processes (Prenter *et al.*, 2004). Aliens often leave their parasites behind because the introductions occur in life stages that do not carry infections or carry only few infections that go lost post-recruitment, e.g. larval stages (Cribb *et al.*, 2000; Torchin *et al.*, 2002). Even when a transport of adults occur, the colonization of a relatively low number of individuals reduces the ability to infest new hosts (Torchin *et al.*, 2002). Thus, invasive species have an advantage over native species in terms of a lower parasite burden (Torchin *et al.*, 2003). When species are however introduced with their parasites, those parasites can spill over to native species and become themselves invasive (Goedknecht *et al.*, 2016). Parasites might be more virulent on native hosts because they have no co-evolutionary history (Fassbinder-Orth *et al.*, 2013) and can lead to disease emergence (Goedknecht *et al.*, 2016).

Generally, by combining both, direct and indirect effects, parasites bear the risk of broadly affecting native species. Assuming that the ecological amplitude of the parasites effect can be proportional to the number of host species affected; the effects of parasites with a complex life cycle can be more wide reaching (Thompson *et al.*, 2005). Nevertheless, (though not always) the effects of parasites with a direct lifecycle can be less sensitive to environmental changes as there is no risk that one of their obligate host species will go extinct (Koh *et al.*, 2004; Molnár *et al.*, 2013). Furthermore, parasites with a direct life cycle are more likely to become invasive, because for parasites with a complex multi-host life cycle the suitable intermediate hosts are often missing (Lymbery *et al.*, 2014). One example of a parasite that spilled over to native blue mussels *Mytilus edulis* is the invasive intestinal copepod *Mytilicola intestinalis*. It has a direct life cycle and is a suitable model to study direct and indirect effects of invasions.

### 3. The *Mytilicola intestinalis* – *Mytilus edulis* parasite-host system

#### 3.1. Bioinvasion of *Mytilicola intestinalis*

The parasite *Mytilicola intestinalis* successfully invaded the North Sea. It was first described from *Mytilus galloprovincialis* at the Gulf of Trieste in the Adriatic Sea in 1902 (Steuer, 1902). The transportation of infected mussel hosts by ship likely caused the introduction of *M. intestinalis* to northern Europe Fig. I.2., with the parasite subsequently spilled-over to native blue mussels (Goedknecht *et al.*, 2017). Although, *M. intestinalis* was occasionally found in other hosts, the parasite is very host-specific and mainly occurs in *Mytilus edulis* in the German Wadden Sea.

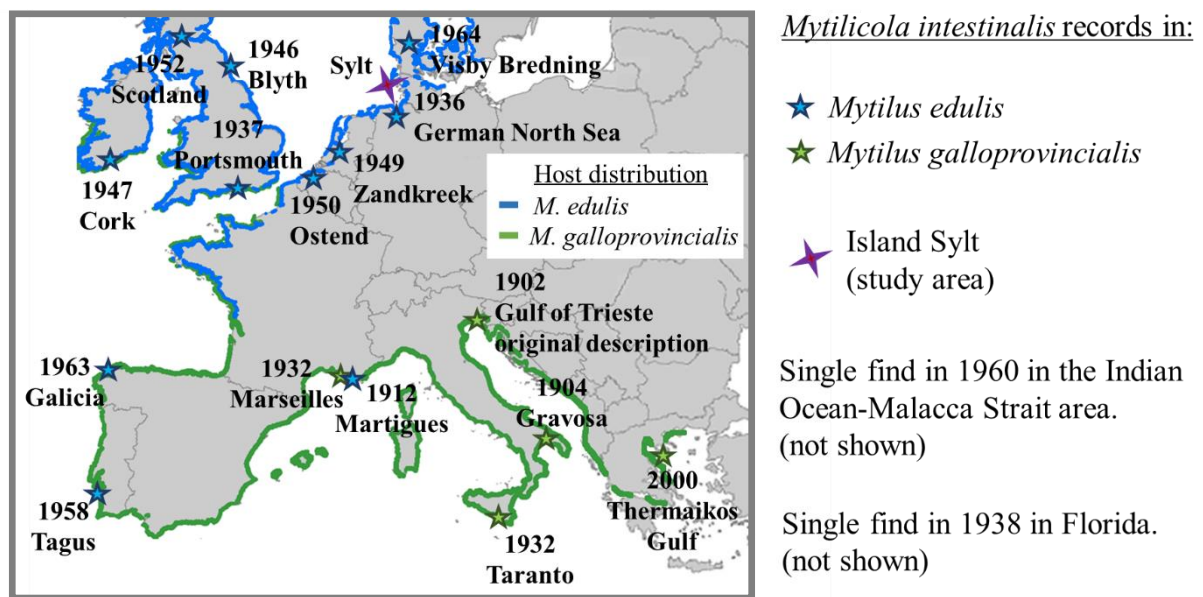


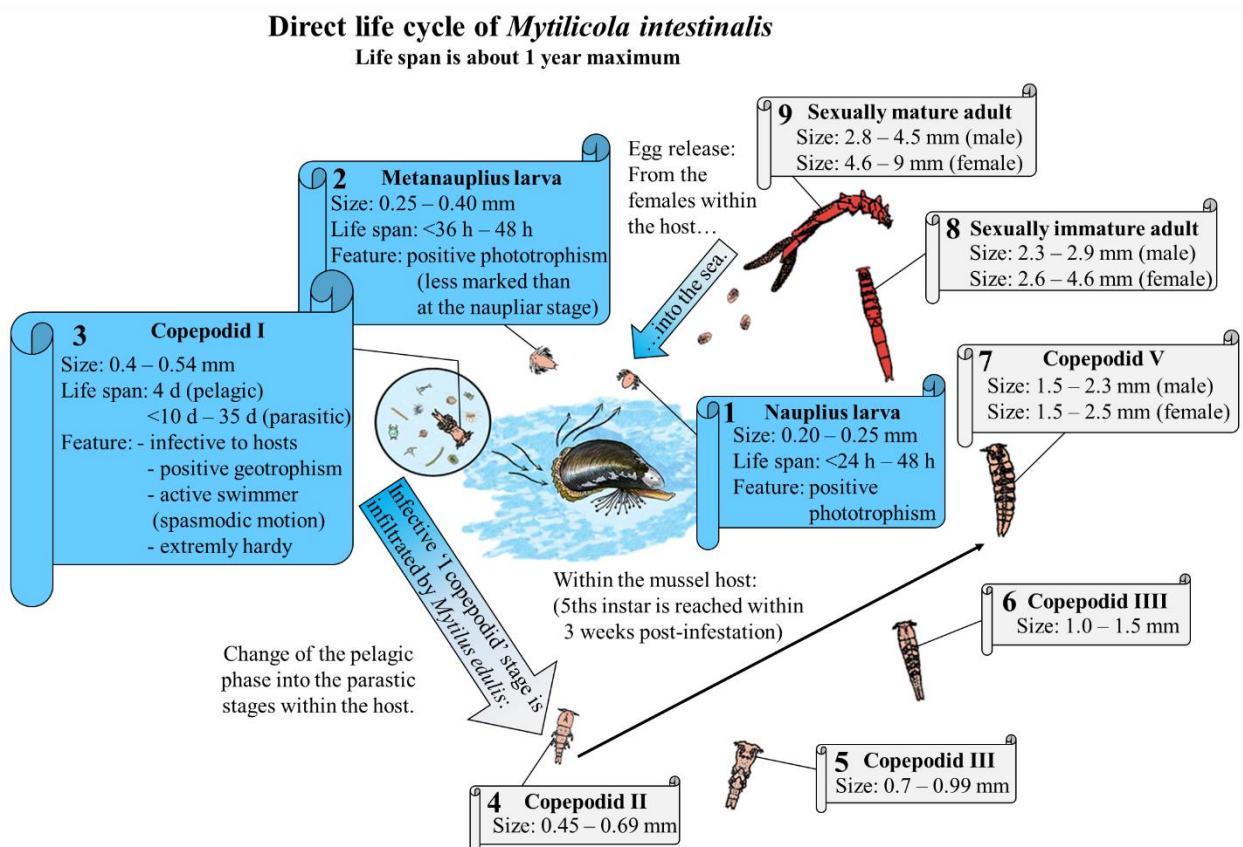
Figure I.2.: Distribution map of *Mytilicola intestinalis*.

The in Fig. I.2. shown data are compiled from the following references: (Andreu, 1963); Thomas (1952) private communication in (Bolster, 1954); (Caillol *et al.*, 1914); (Caspers, 1939); (Cerruti, 1932); Beanland (1937) as well as Harding (1937) personal communication in (Cole & Savage, 1951); (Ellenby, 1947); (Korringa, 1950); (Leloup, 1951); Cole (1947) private communication in (Meyer & Mann, 1950); (Monod & Dollfus, 1932); Wilson in (Pearse & Wharton, 1938); (Rayyan *et al.*, 2004); (Steuer, 1905); (Steuer, 1902); (Theisen, 1964); (Vilela & Monteiro, 1958); (Wickstead, 1960).

In general, the two main factors that govern the natural distribution distance of *M. intestinalis* are water currents and time the nauplii stay at the pelagic stage (Bolster, 1954; Hockley, 1951). However, mainly due to anthropogenic ship traffic, the invasive distribution of *M. intestinalis* is radial and the infection of new hosts may proceed in a geometrical progression (Bolster, 1954; Meyer-Waarden & Mann, 1954b), Fig. I.2. The introduction ways of *M. intestinalis* were partly like those of *Crepidula fornicata* and *Eriocheir sinensis* (Bolster, 1954; Meyer-Waarden & Mann, 1954b).

### 3.2. Direct life cycle of *Mytilicola intestinalis*

*Mytilicola intestinalis* is a parasitic cyclopoid copepod that lives in the intestine of shellfish of the infraclass Autolamellibranchiata. It modified its morphology to attach to the intestinal tract of its mussel host during the parasitic phase of its life cycle (Crites, 1962). In its very first life stages it is pelagic with a reduced number of nauplii stages; 2 as opposed to 6 (Elgmork & Langeland, 1970) significantly shortening the duration of the pelagic phase, Fig. I.3.



**Figure I.3.:** Direct life cycle of *Mytilicola intestinalis* showing the embryonic, free-living and parasitic phases.

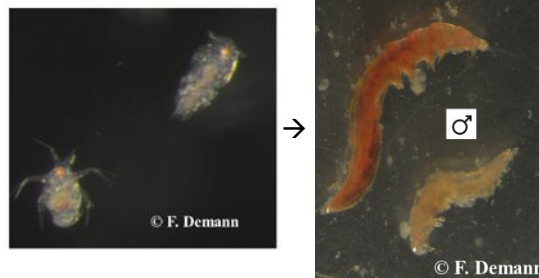
Given life spans are approximate values that can differ for various temperature conditions.

Pictures and data shown in Fig. I.3. are obtained and modified after (Bolster, 1954); (Caspers, 1939); (Davey & Gee, 1988); (Gee & Davey, 1986b); (Grainger, 1951); (Hockley, 1951); <http://www.coopzeitung.ch/> (2017); <http://weichtiere.at/> (2017).

This short duration and reduction of free-living nauplii stages reduce the possibility of being washed out from the host habitat before they reach infectivity (Williams, 1969b) and thus, could maximize the likelihood of re-infestations to new hosts (Gee & Davey, 1986b). This also implies that *M. intestinalis* does not naturally expand over long distances. Nevertheless, within the first two developmental stages the biological spread is highest (Bolster, 1954). Due to their positive phototactic response, nauplii stages occur mostly within the upper water column where the currents are strongest (Bolster, 1954; Korringa, 1968). After the 'nauplius' and 'metanauplius' stage, the 'copepodid I' stage is reached, which is

infective to hosts, Fig. I.4. In contrast to the nauplii stages, the ‘copepod I’ formed a hook at the antennae that enables the parasites to cling the gut wall of the host after being infiltrated (Grainger, 1951; Hockley, 1951).

**Figure I.4.:** Nauplia larva (less than 24 h old) and ‘copepodid I’.



**Figure I.5.:** Adult *M. intestinalis*. Above: carry-over from the wild Below: experimentally reared (younger than above) The appendages are clearly visible at both stages.

### 3.3. Within the host

After moulting through four copepodid stages (copepodid II-V) pre-adult stage (which is sexually immature), is reached shortly before the sexually mature adult stage (Gee & Davey, 1986b). Further molts only increase body length but do not lead to morphological changes (Caspers, 1939). The mature adults have a blood-red elongated body with short appendages, Fig. I.5. It looks worm-like, hence the term ‘red worm disease’. In terms of growth, females reach bigger sizes than males and can carry two egg sacs when ovigerous, Figs. I.6./I.7. According to Campbell (1970), the approximate length of females spans maximally to 10 mm. The copepod females carry egg sacs with about 20 to 200 eggs per sac with bigger females generally having more eggs (Davey *et al.*, 1978).



**Figure I.6.:** Adult male.



**Figure I.7.:** Adult ovigerous female.

Within the egg sacs, the embryonic phase starts after the last cell division with embryos already showing a median eye (Gee & Davey, 1986b). The nauplii are singly enclosed in a membrane and the female sheds them into the gut cavity from where they are released into the environment (Grainger, 1951); Fig. I.3. The cycle newly begins when embryos leave the host, develop and hatch as naupliar; *M. intestinalis* thus uses only one host for all its life.



The natural life cycle of *M. intestinalis* is tightly regulated by temperature, which is likely to govern the rates of development of the parasite rather than being a threshold for the onset or cessation of specific developmental stages (Davey *et al.*, 1978; Gee & Davey, 1986b; Williams, 1969b). This holds true for all developmental life stages, although all stages have a wide ecological range (Korringa, 1968).

### 3.4. Movements in the host's gut

Within the host's gut, parasites are positioned with the head oriented in the direction of incoming food, (Hockley, 1951). Using their thoracic appendages; the parasites press their ventral surface against the wall of the host's intestine, in a way that allows some of the oncoming food to pass through the oral surface of the parasite (Hockley, 1951). The maxillules of *M. intestinalis* are relatively wide apart and contain a row of ridges with each forming a small tooth that is used to attach to the gut wall and maintain position within the host (Hockley, 1951). The very first parasitic stages occur in the stomach (Gee & Davey, 1986b) and move downward the gut towards the rectum when maturing (Davey & Gee, 1988; Dethlefsen, 1972; Grainger, 1951) to accommodate increased body size and the need to release the ripe eggs (Davey & Gee, 1988). These movements might cause metaplastic changes within the cells of the gut epithelium of the host (Moore *et al.*, 1978). For a successful reproduction, individuals of one female and one male same age must be present within the same host (Bolster, 1954; Korringa, 1968). When an adult parasite detaches from the host (Fig. I.8.), it cannot re-occupy a host because of the shortened appendages (which is poorly adapted to swimming) and a body size that is too big for an uptake by a host (Davey & Gee, 1988; Davey *et al.*, 1978; Grainger, 1951).



**Figure I.8.:** *M. edulis* expelling a parasite (both alive).

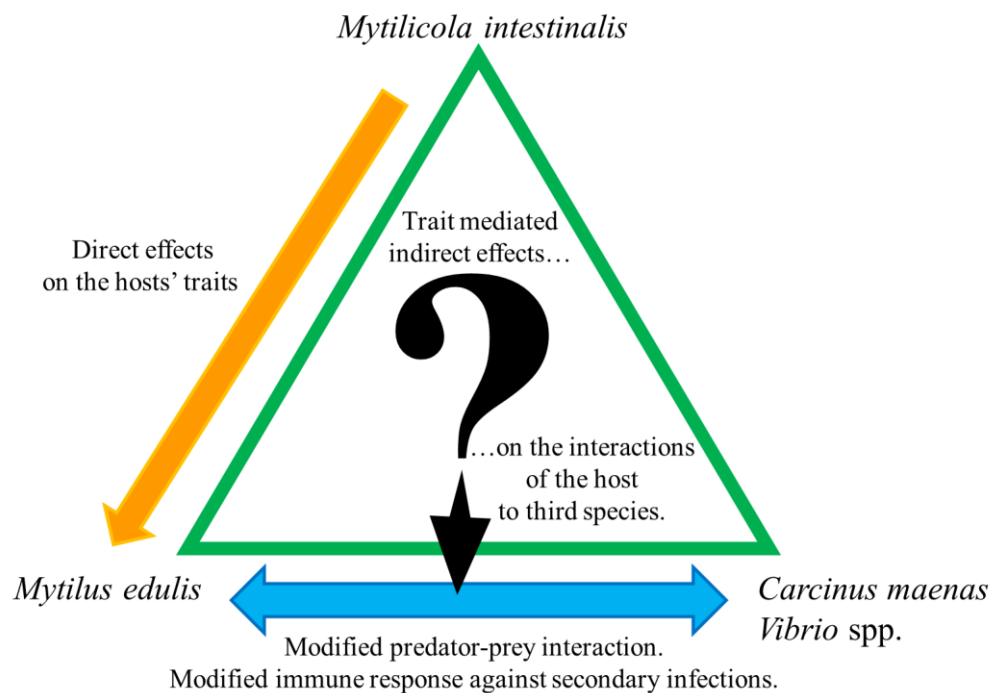
### **3.5. Effect of *M. intestinalis* on its host**

The collapse of mussel fishery in Germany, France and the Netherlands during 1949 and 1984; resulted from mass mortalities of mussels, which was attributed to infection by *M. intestinalis* (Blateau *et al.*, 1992; Korrynga, 1950; Meyer & Mann, 1950). However, direct involvement of the parasite in the high mussel die offs was never proven, because mussel deaths during these mortality events were not screened for other ‘hidden infections’ (Lauckner, 1983). Nevertheless, since the first mortality events, *M. intestinalis* has been a major focus of marine studies, but with its ecological impact on the host is very contradictory (Korrynga, 1968; Lauckner, 1983). Some authors classified it as a harmless commensal of blue mussels (Davey, 1989), while others found considerable effects on mussel condition, (Cole & Savage, 1951; Meyer-Waarden & Mann, 1954a).

The effects of *M. intestinalis* are the focus of this thesis. In other words, the impacts of infection reported in the literature are covered in detail within the following chapters (I-III). While there is ample evidence for direct tissue damage caused by attachment to the gut wall, the consequences for host fitness are more controversial and might additionally depend on temperature and duration of infection.

#### 4. Thesis outline

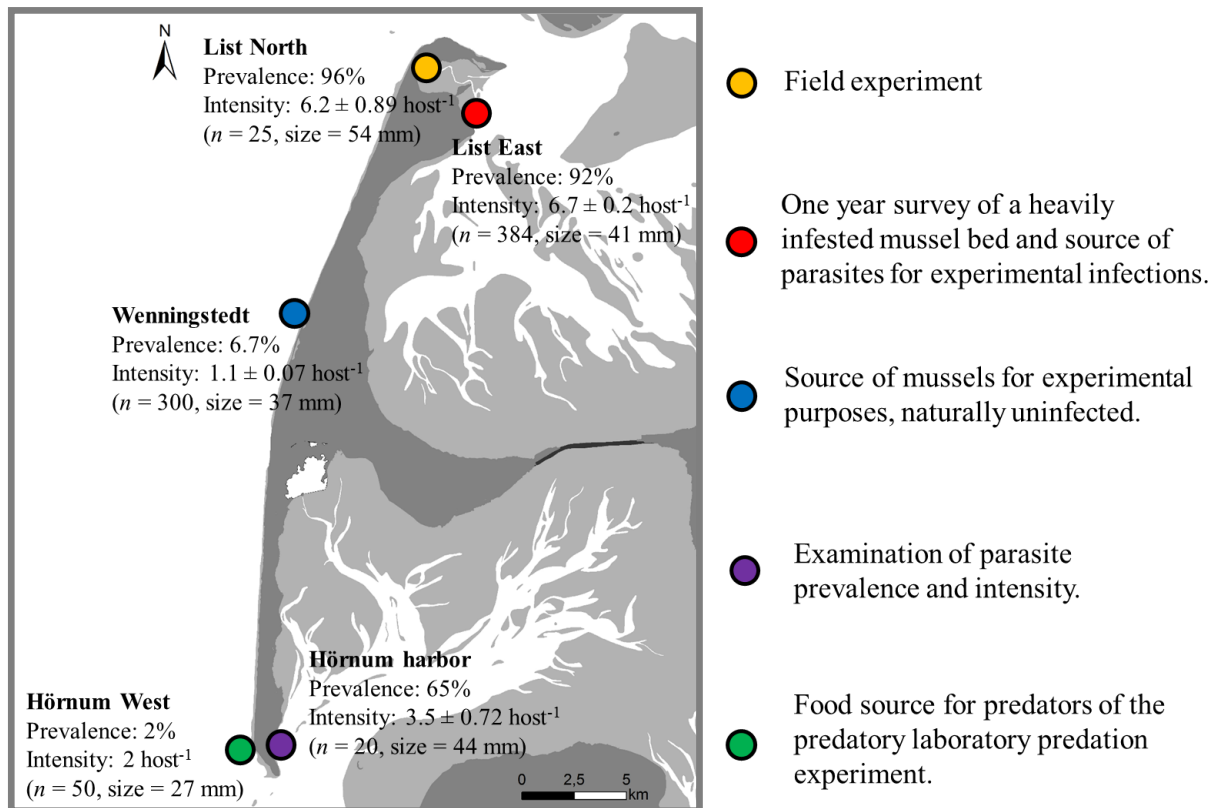
Direct and indirect effects are widespread in nature, ramify throughout species communities and have a far-reaching ecological relevance (Dunn *et al.*, 2012; Walsh, 2013). Whereas parasites will always have a direct effect on their host, they can also have indirect effects on native biota. Indirect effects of parasites to other species can be greater than the direct effects on the host (Granroth-Wilding *et al.*, 2015). Usually, indirect effects require the presence of intermediate species that are often parasites, Fig. I.9. The indirect interaction of intermediate parasites with non-host species are pivotal in structuring communities, (Hatcher *et al.*, 2014; 2012) and the importance of parasites in mediating indirect effects within invasion processes is increasingly recognized (Goedknecht *et al.*, 2016). Co-invading parasites as disease-mediating species take a special part within invasion processes for example by changing pathogen-dynamics (Hatcher *et al.*, 2012; Strauss *et al.*, 2012). There are many examples of parasites and pathogens indirectly causing high mortalities on native species via the process of invasive spillover, (Gozlan *et al.*, 2005; Tompkins *et al.*, 2003).



**Figure I.9.:** Schematic representation of direct and indirect effects in the *M. intestinalis* – *M. edulis* system.

The present thesis addresses the direct and indirect effects of *M. intestinalis* on native blue mussels. The overall aim is to answer the question if *M. intestinalis* affects its host and if this effect is subject to further changes like temperature. By combining results of several laboratory and field experiments, the effects of a parasite invasion on the newly acquired native host will be investigated in unprecedented details. The simultaneous assessment of multiple facets of direct and indirect effects will aid to estimate the overall impact of parasite invasions.

Figure I.2. shows the position of the study area. For the purpose of this study, I sampled different mussel beds around the island Sylt. Figure I.10. gives detailed information on the sampling points and use of collected mussels as well as the several parasite incidences.



**Figure I.10.:** Sampling locations around the island of Sylt (German Bight, North Sea).

The subtidal area is in white, tidal flats in light grey and the land above spring high tide level in dark grey. The next chapters give the exact position of the intertidal sampling locations.

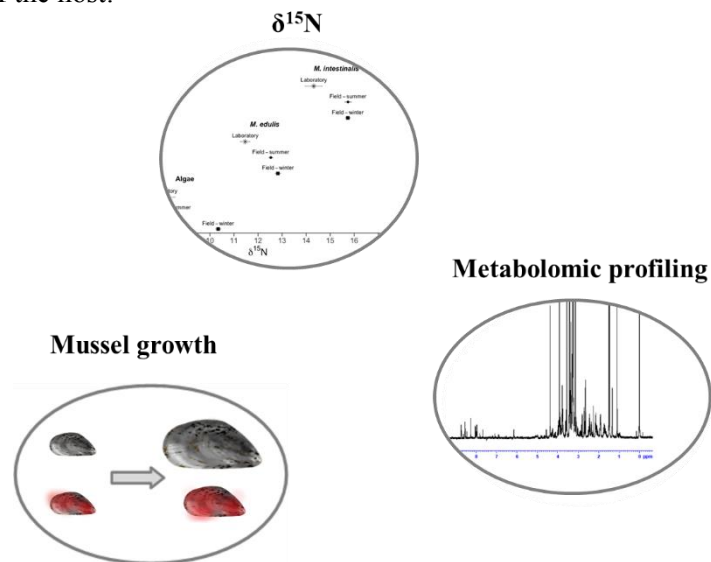
Source: Topographic Wadden Sea GIS map 2015, issued by the Schleswig-Holsteins' Government-Owned Company for Coastal Protection, National Parks and Ocean Protection – National Park Authority, Tönning; made available by Jörn Kohlus.

First, I investigated several direct effects of *M. intestinalis* by laboratory and field experiments, (Chapter I). Subsequently, I concentrated on the (trait-mediated) indirect effects of *M. intestinalis* on the predator-prey interaction of *M. edulis* and the shore crab *Carcinus maenas* (Chapter II). Furthermore, I looked for the indirect effects of *M. intestinalis* on the disease interaction between *M. edulis* and pathogenic *Vibrio* spp. (Chapter III).

## Chapter I

In this chapter, the direct effects of parasite infection on the mussel host will be addressed in experimental setups (both in the field and in the laboratory). Direct effects on mussel growth and condition were assessed on multiple temporal scales applying several techniques. This included stable

isotopic analysis to determine the main food source of the parasite (i.e. host vs. host food), metabolomic profiling to assess shifts in energy allocation and long-term mussel growth experiments in the lab and in the field, Fig. I.11. To extrapolate these results to the natural situation in the wild, I also conducted a survey of parasite infection throughout a whole year to determine how these effects will be distributed over the life cycle of the host.

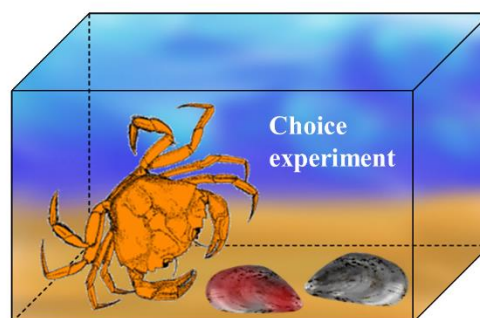


**Figure I.11.:** Schematic diagram of the experimental methods. Red colored mussels are parasitized with *M. intestinalis*.

I could show that the seasonal cycle of parasite infestation follows a distinct pattern correlated to temperature. Since *M. intestinalis* feeds on mussel tissue, and thereby changes the metabolomic profile this might reduce the mussels' condition. Therefore, *M. intestinalis* clearly exerts direct effects on its host by limiting the available resources for important life history traits like growth.

## Chapter II

Parasites can have strong ecological impacts via indirect interactions with non-host species (Hatcher *et al.*, 2014). Trait-mediated effects on the host that change predator-prey interactions may be crucial to population dynamics (Hatcher *et al.*, 2014). Those indirect effects mediated by parasites can be pivotal even if parasites have a negligible virulence (Hatcher *et al.*, 2014).

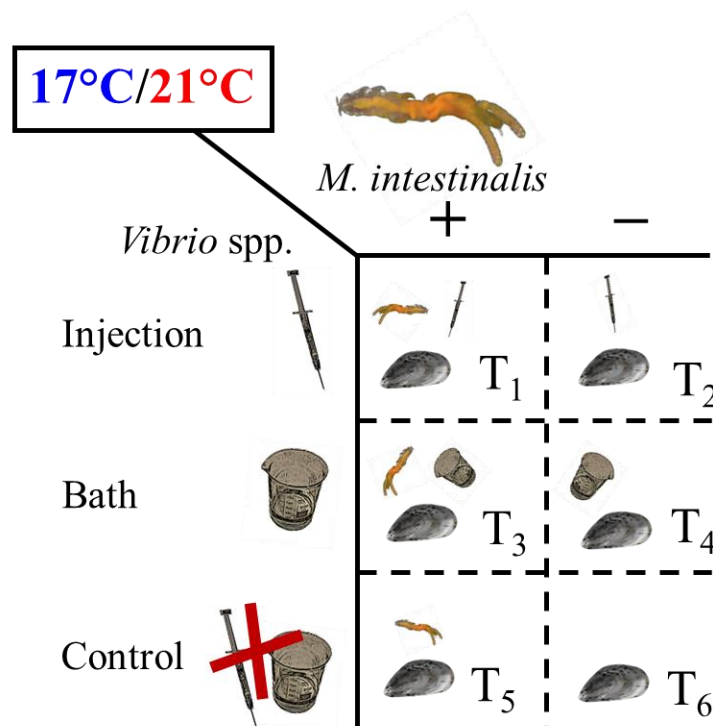


**Figure I.12.:** Schematic diagram of the experimental predation experiments. Red coloration of mussels indicates infection with *M. intestinalis*.

Within chapter II, I investigated the predation behavior of the shore crab *Carcinus maenas* when offered parasitized and non-parasitized mussels. In controlled prey choice experiments, crabs should choose between parasitized and non-parasitized mussels and I observed the prey preference of crabs, Fig. I.12. The results show that shore crabs prefer non-parasitized mussels in summer, when the parasite has not yet reached sexual maturity. When parasites matured in autumn, shore crabs preferred parasitized mussels indicating that *M. intestinalis* can indirectly affect species interactions although not directly liaise with the interacting parties.

### Chapter III

Intestinal parasites can affect their host by causing lesions within the hosts gut (Hiepe *et al.*, 2006). I investigated the immune response and the resistance of *M. edulis* against secondary infections by measuring the phagocytosis rate of mussels as well as the *Vibrio* load for different infection scenarios. Therefore, I combined parasite infection (control / infected) with *Vibrio* infection (control / injection / bath challenge). Figure I.13. shows the different infection treatments (T<sub>1</sub>–T<sub>6</sub>). I conducted this experiment under two different temperature settings (ambient and raised temperatures), thus, shifting the thermal optimum from mussels to bacteria.



**Figure I.13.:** Combination of different *Vibrio* and parasite infection treatments (carried out at 17 °C and 21 °C).

With the results, I could show an inhibited phagozytosis rate within parasitized mussels although *M. intestinalis* is not directly interacting with the hemocytes of *M. edulis*. Altogether, parasitized mussels showed a higher *Vibrio* spp. load, leading to a higher mortality over time especially at higher temperatures. Thus, I could show that *M. intestinalis* has an indirect effect on the pathogenesis of a

secondary infection. Although this might weaken the immune response of *M. edulis*, it is likely that further stressors are needed to invoke high mortality rates.

Overall, I could show that *M. intestinalis* has direct and indirect effects on native blue mussels and their interacting species. The parasite induces alterations in the immune response of the mussel and alters the energy budget. The results of this study clearly demonstrate that *M. intestinalis* is not a harmless commensal to *M. edulis* but a real parasite exploiting its host. Thus, the mussel permanently uses energy to cope against the effects of the parasite. This energy might lack for other basic functions like mussel growth. Although mussels can potentially evolve resistance against infection (Feis *et al.*, 2016), infected mussels are at a clear disadvantage. How future climatic conditions might change the interaction of parasite and host within this system remains an open question.





## **II. Chapters I-III**

# **Chapters I-III**



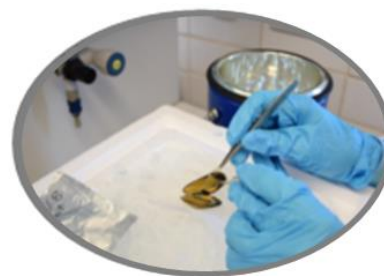
**Chapter I**

**Direct effects of a long established invasive parasite on native blue mussels**

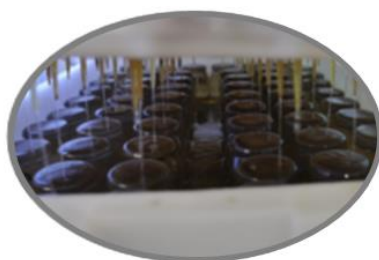
$\delta^{15}\text{N}$



**Metabolomic profiling**



**Mussel growth**





## Direct effects of a long established invasive parasite on native blue mussels

Felicitas Demann<sup>a</sup>, Gisela Lannig<sup>b</sup>, Christian Bock<sup>b</sup>, Benoit Lebreton<sup>c</sup>, Christian Buschbaum<sup>a</sup>, K. Mathias Wegner<sup>a1</sup>

### Abstract

*Mytilicola intestinalis* is an invasive intestinal copepod that was introduced into the North Sea in the 1930ies and is now strongly associated with blue mussels *Mytilus edulis* in the Wadden Sea. Since 1949, the parasite *M. intestinalis* was blamed for blue mussel mass mortalities in Germany, the Netherlands and France and therefore became of interest for many biological and ecological studies. It remains unsolved if the parasite was indeed responsible for mussel mortalities or if it only acts as a commensal to mussels with no harmful effect on its host. Most of the *Mytilicola*-studies were done under field conditions with an uncontrolled number of parasite infections and the correlative nature of the results might confound the effect of the parasite on its host. Due to its direct life cycle, *M. intestinalis* offers a convenient handling under laboratory conditions. Despite the potential hazard and the suitability of such host/parasite-systems to answer ecological questions, only a few studies explicitly make use of this invasive parasite in controlled experiments. Here we apply several methodological approaches to assess different direct effects of *M. intestinalis* on blue mussels by combining controlled infection experiments in the laboratory and in the field with background infection data from a one-year field survey. Our results clearly show that the parasite feeds on the hosts' tissue and the novel application of <sup>1</sup>H-NMR (nuclear magnetic resonance) analysis revealed that mainly metabolites of the amino acid metabolism were affected in some tissues suggesting that. Permanent coping with the parasite is energetically demanding. This might cause a lack of resources for other basic processes and might result in reduced condition for parasitized mussels and ultimately reduced growth. Since these direct effects only became apparent in our controlled experiments, it is important to include such multidisciplinary investigations to evaluate the impacts of invasive parasites on native hosts.

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### 1. Introduction

The transmission of parasites within invasion processes might crucially influence the success of marine bioinvasions and can have profound ecological implications for native biota (Prenter *et al.*, 2004). During a biological invasion, an introduced species may lose its parasites thus, having a competitive advantage over native species (Torchin *et al.*, 2001). Aliens may also acquire parasites from the new range or might introduce novel parasites to native hosts via the process of parasite spillover (Dunn, 2009; Goedknecht *et al.*, 2017). Often, invasive parasites can cause a greater impact on the native host than on the original invasive host (Mastitsky *et al.*, 2010). We consider this latter case of parasite invasion using the parasite *Mytilicola intestinalis* as model system to study direct effects on its new native blue mussel hosts. Generally, the direct effects of parasites are diverse and can affect the hosts' traits, density, behavior or reproductive output (Chiaverano *et al.*, 2015). Moreover, parasites often affect their hosts in multiple ways forming a complex system of direct effects simultaneously acting on the host. Direct lifecycle parasites like *M. intestinalis*, that we used in our study, are methodologically particularly suitable for studying those direct effects. Hence, it is possible to observe the direct effects of invasive parasites on the hosts over long uninterrupted experimental periods.

*M. intestinalis* is an invasive parasite that was introduced into the North Sea in the 1930ies and is now strongly associated with blue mussels *Mytilus edulis* in the Wadden Sea (Caspers, 1939; Pogoda *et al.*, 2012). The direct effects of *M. intestinalis* on the host are very controversially discussed in the literature, (Lauckner, 1983). On the population level, the parasite was blamed to cause mass mortalities in the Netherlands, Germany and France between 1949 and 1984 (Blateau *et al.*, 1992; Korrynga, 1950; Meyer & Mann, 1950). Mussels that died during those mortality events were not examined for other pathogens, (Lauckner, 1983). There has been no reported event of blue mass mortalities that was linked to parasite infection since the last mortality event in France. However, many studies suggest that the mussels' condition is affected only by high parasite loads combined with adverse environmental conditions (Gee *et al.*, 1977; Paul, 1983; Theisen, 1987). On the other hand, other authors found that heavy parasite infestations did not harm mussel populations (Davey & Gee, 1988; Dethlefsen, 1975) and often stated that individual mussels did not suffer from high parasite infection intensities (Bolster, 1954). The question arose if *M. intestinalis* is a real parasite (Davey, 1989).

Commonly, the direct host alteration is the main criterion defining a parasite as independent lifeform, (Hiepe *et al.*, 2006). This alteration or harm can occur through three main directions. These are food deprivation, metabolic damage and the energetically costly mechanical impact on the hosts' tissue, (Hiepe *et al.*, 2006). *M. intestinalis* is a relatively large intestinal parasite and thus, has the potential to affect its host along all three dimensions. Within this study, we tried to explore the main direct effects of this parasite on mussels by combining different methodological approaches addressing all three directions. Those experiments comprise investigations on the nitrogen isotope values of mussels,

parasites and mussel food sources, on the metabolic profile of parasitized and non-parasitized mussels and on the growth and condition of mussels with different infection intensities. Moreover, we used controlled infection intensities of the parasite thus, minimizing possible interferences of natural background variation. The experiments were conducted under both, laboratory and field conditions and at different seasonal times of the year providing novel insights into the direct effects of *M. intestinalis*. Additionally, we monitored the parasites incidence of a natural infested mussel bed over one year and determined the monthly parasite prevalence and intensity. Thus, we could assess the experimentally observed direct effects of *M. intestinalis* in the view of natural infection intensities. Generally, the extent of the identified direct effects of a parasite on a host can vary due to seasonal variations in parasite intensity and life cycle of the mussel host. Hence, we could draw conclusions about the seasonal time when the parasites' direct effects are expected to be strongest on the host. Using a multidisciplinary approach, we tried to close the gap of how *M. intestinalis* affects its host on multiple scales and can potentially extrapolate the energetic costs for the mussels over time.

## **2. Materials and Methods**

### **2.1. Biological material**

For all the experimental purposes, we used mussels from the west side shore of the island of Sylt in the northern Wadden Sea (German Bight, North Sea). Here, mussels grow on groins (Wenningstedt; 54°56.522' N, 8°18.946' E) and usually show very low infection levels. The sampled mussels from here ( $n = 300$ , size = 37 mm) had a prevalence of the parasite of 6.7 % and a mean intensity of  $1.1 \pm 0.07$  parasites infected host<sup>-1</sup>.

A littoral mussel bed located at the east side of the island of Sylt (List east; 55°01.331' N, 8°26.456' E), provided a naturally heavily infested parasite source for the experimental infections of all the field and laboratory experiments conducted within this study.

### **2.2. Experimental infections and maintenance of mussels**

We used uninfected and experimentally infected mussels for laboratory and field experiments investigating growth, stable isotope signature, condition measurements and metabolic profiles. Mussels from the uninfected source population were separated from each other, all attached epibionts (e.g. barnacles) were removed and the mussels were rinsed with tap water. For maintenance, mussels were held singly in 200 mL plastic bottles at a constant temperature of 17 °C. Each bottle received its own supply of ambient, running seawater. The water was filtered before entering the bottles over a 50 µm mesh to prevent subsequent cross-infections by spread of small *M. intestinalis* nauplii, which size is 0.2 mm (Gee & Davey, 1986b). Mussels were fed with a constant supply of instant algae Isochrysis 1800™ (ReedMariculture Inc., U.S.A.). Half of the mussels were experimentally infected with

*M. intestinalis* after an acclimatization phase to laboratory conditions. Infection was done by incubating embryo sacs of *M. intestinalis* from ovigerous females. Egg sacs were put individually into wells, (3.5 mL) of 24 cell culture plates filled with sterile filtered seawater (0.45  $\mu\text{m}$ ) and incubated at ambient temperature. Sterile filtered seawater was used to maximize rearing success of embryos as this is inhibited by bacterial pollution (Caspers, 1939). Naupliar larvae hatched within the time period of one day to several days, depending on the developmental stage of the egg-sacs when collected. Copepodids were used to infect mussels, when the infective first copepodid stage was reached. Sets of 25 or 50 copepodids – depending on the experimental purpose – were transferred by a glass Pasteur pipet into 200 mL plastic bottles (explicit number of copepods is given for each experiment in the respective section). One mussel was put into each bottle and then covered with filtered seawater. Mussels were kept overnight (at least 6 hours) in contact with the copepodids to ensure that more than 75 % of the copepodids were ingested by a mussel (Gee & Davey, 1986a). Uninfected control mussels were treated in the same way but no copepods were added to the bottle.

### 2.3. Stable isotope analyses

Differences of  $\delta^{15}\text{N}$  values between *M. edulis* food sources, *M. edulis* and *M. intestinalis* were determined based on two field surveys, one in winter (January 2015) and one in summer (July 2016), and one laboratory experiment. The seasonal assessments, as well as the *in vitro* one, allowed us to determine if parasitic behavior change depending on variations of the quality of the suspended particulate organic matter, that we assessed by computing C/N ratios. For field surveys, suspended particulate organic matter (i.e. mussel food resource) and mussels were collected at List east. Suspended particulate organic matter was collected by filtering the surface seawater on pre-combusted (450°C, 4 hours) glass microfiber filters (GF/F Whatman™). Mussel soft tissues were dissected (i.e. removal of the gut content) and parasite samples were collected. All samples were freeze dried and stored at -80 °C until further analysis. For the laboratory experiment, mussels were collected in April 2016 at Wenningstedt, then infected with 25 copepodids each and maintained for 2 ½ months following the methods described in section 2.2. Mussel and parasite samples collected at the end of the experiment were then treated on a similar way as those from the field surveys. Suspended particulate organic matter in the experimental tanks was collected by filtering the incoming water on pre-combusted glass fiber filters, and then freeze-dried.

Mussel and parasite samples were ground to a fine powder using a ball mill (Retsch® MM 301). Mussels were analyzed individually and all parasite individuals that were dissected out of one mussel were grouped together. All samples were analyzed using an elemental analyzer (Flash EA 1112, Thermo Scientific, Milan, Italy) coupled with an isotope ratio mass spectrometer (Delta V Advantage with a Conflo IV interface, Thermo Scientific, Bremen, Germany) at the LIENSs stable isotope facility of the University of La Rochelle, France. Results are expressed in the  $\delta$  notation as deviations from a standard



(N<sub>2</sub> in air) following the formula:  $\delta^{15}\text{N} = [(R_{\text{sample}}/R_{\text{standard}}) - 1] \times 10^3$ , where R is <sup>15</sup>N/<sup>14</sup>N. Calibration was done using reference materials (IAEA-N2, -NO-3, -600). Analytical precision based on the analysis of acetanilide (Thermo Scientific) and peptone (Sigma Aldrich) used as laboratory internal standards was <0.15 ‰.

#### 2.4. Untargeted NMR based metabolic profiling

Specimens of the blue mussel (3.6–5 cm shell length) were collected at Wenningstedt in July 2015 to evaluate the impact of *M. intestinalis* infection on the metabolic profiles of specific tissues of *M. edulis*. In the laboratory, half of the mussels were infected with 50 *M. intestinalis* copepodids each (see the method described in section 2.2.) to get two different mussel groups (non-parasitized and parasitized mussels). After an infection period of 4 ½ months under laboratory conditions (as described in section 2.2.), posterior adductor muscle, foot, gills and mantle tissues of 20 mussels (10 replicates for both mussel groups) were sampled. Each mussel was checked for the infection grade and the number of copepodids counted. Tissue samples were immediately freeze-clamped in liquid N<sub>2</sub> and stored at –80 °C for further analysis. Polar metabolites of the frozen tissue samples were extracted using the methanol dichloromethane extraction technique for NMR samples described by (Wu *et al.*, 2008) after a slightly modified protocol for small samples presented in (Tripp-Valdez *et al.*, 2017). Frozen tissue sample, (≈ 100 mg) was homogenized in 400 µL of methanol and 85 µL of deionized water for 20 s at 6.000 rpm and 4 °C using a Precellys 24 (Bertin Technologies, France). Afterwards 400 µL of chloroform and 440 µL of deionized water were added and the sample was incubated on ice for additional 10 min. After centrifugation (10 min at 3.000 rpm and 4 °C), three phases were observed. The upper methanol layer containing polar metabolites from the cytosol was transferred into a 2 mL tube and dried at room temperature by vacuum centrifugation overnight (RVC 2–18, Martin Christ Freeze dryers, GmbH, Germany). For untargeted metabolic profiling based on <sup>1</sup>H-NMR spectroscopy, dried samples were resuspended with D<sub>2</sub>O (3x volume of tissue weight) containing 0.1 % trimethylsilylpropanoic acid (TSP, Sigma-Aldrich, St. Louis, USA), an internal reference marker acting as a chemical shift reference (≈ 0.0 ppm). All <sup>1</sup>H-NMR spectra were acquired with a 9.4 T Avance III HD 400 WB spectrometer equipped with high resolution magic angle triple tunable <sup>1</sup>H-<sup>31</sup>P-<sup>13</sup>C probe (HRMAS) using TOPSPIN 3.2 software for acquisition and processing (Bruker Biospin GmbH, Germany). Before the NMR measurements, 70 µL of the resuspension was transferred to a 4 mm standard HRMAS zirconia rotor. A Call-Purcell-Meiboom-Gill (CPMG) sequence with water saturation were used for the acquisition of metabolic profiles at 20 °C using the following parameters: flip angle 90°, acquisition time 4.01 s, relaxation delay 4 s, sweep width 8.803 Hz, 64 scans with 4 dummy scans. An exponential line broadening of 0.5 Hz was applied prior Fourier transformation and a zero-filling of 64k was used for processing. All spectra were manually corrected for phase and baseline shifts and calibrated using TSP as internal standard at 0.0 ppm. Specific metabolites were quantified using Chenomx NMR Suite 8.1 software (Chenomx Inc., 2014). Signals were assigned to specific metabolites using the data bank

provided by Chenomx in combination with literature data (Ellis *et al.*, 2014; Podbielski *et al.*, 2016; Zittier *et al.*, 2015).

### **2.5. Mussel growth experiment – Field**

A field experiment was conducted from June 2015 to May 2016 to explore long-term effects of *M. intestinalis* infestation on both, mussel shell increment and condition. Here, blue mussels were collected at Wenningstedt in May 2015 ( $n = 72$ ). In the laboratory, the half of mussels were infected with 25 *M. intestinalis* each (see method in section 2.2.). After the infection procedure and just before starting the experiment, mussel fresh weight was determined to the nearest 0.0001 g using a precision balance and mussel length was measured to the nearest 0.01mm using a digital caliper. Weight and length measurements were done six-times and the mean value used for statistics. In the mid of June 2015, the infected and the uninfected mussels were put individually into cages (23 x 23 cm plastic net pockets with a mesh size of  $\approx 1$  cm), which were fixed at the experimental site (List north; 55°02.407' N, 8°24.764' E) by using one iron rod for each cage. After an experimental period of one year (i.e. end of May 2016), the experiment was terminated and mussel weight and size were measured again. The mussels were then dissected and the number of parasites was counted. After dissection, we determined the dry weight to calculate mussel condition. Dry weight was measured after drying all items (at 50 °C) until constant weight. The condition index (CI) was determined following the formula  $CI = \frac{DWF}{L^3}$  with *DWF* is the dry weight of the mussel flesh and *L* is the length of the mussel shell (Petersen *et al.*, 2004). For each mussel host we measured the crushing force of the shells. For mussel shell strength measurements, tissue was removed and strength of both valves was individually measured for each mussel to the nearest 0.1 N by using a compression load instrument, which is described in detail in (Buschbaum *et al.*, 2007). This device measured the force required to crack valves, which were positioned laterally between two horizontal plates (inner/open part of the valve towards bottom plate).

### **2.6. Mussel condition experiment – Laboratory**

A laboratory experiment was performed to determine the condition of mussels infested with different *M. intestinalis* intensity. Mussels were collected in April 2016 at Wenningstedt and infected in May with either 25 or 50 copepodids. Therefore, three differently infected mussel groups were used for the experiment ( $n \approx 10$  mussels per group): 1) uninfected mussels, 2) moderately infected mussels with about 25 copepods each and 3) heavily infected mussels with about 50 copepods each. Mussels were held under laboratory conditions according to the method described in section 2.2. for 4 ½ months. Afterwards, the mussel fresh weight and the shell length of the mussels were measured and the number of adult parasites (males and females) was counted for statistics by dissecting the mussels. The dry weight of mussel tissues was determined according to the methods described in section 2.5. and the condition index was also computed as described in section 2.5.

## 2.7. Field survey

A littoral mussel bed located at the east side of the island of Sylt (List east), provided a naturally heavily infested stock to examine the seasonal population dynamics of *M. intestinalis*. For a period of one year between October 2014 and October 2015, we sampled thirty blue mussels *Mytilus edulis* (2.1–6.4 cm shell length) at monthly intervals, to determine the prevalence and intensity of *Mytilicola intestinalis*. After collection, mussels were transferred to the laboratory and weighed to the nearest 0.0001 g and mussel length was measured to the nearest 0.01 mm using a digital caliper. Parasites were then dissected from the intestinal tract of mussels. We recorded the numbers of males, females, juveniles and egg sacs and photographed all individuals to measure length to the nearest 0.01 mm with the image analysis software Leica QWinV3 ‘Digital image processing and analysis software for professional microscopy’, (Leica Microsystems, Switzerland, Ltd, 2006). We categorized juveniles from >0.0 to 2.7 mm, females from 1.5 to 9 mm and males from 1.5 to 4.5 mm (Gee & Davey, 1986b). Males and females were distinguished via morphological traits (broadened abdominal segment in females) or by the presence of eggs. Furthermore, juvenile and adult parasites were distinguished via intensity of coloration of hemoglobin that gradually increases after the first adult stage (Grainger, 1951; Korrynga, 1968). Juveniles were usually almost transparent. We excluded parasites that were damaged during the dissection process from length measurements (<10 %). The dry weight of the mussel flesh was determined separately according to the methods described in section 2.5. The condition index was computed as described in section 2.5. Additionally, we measured the crushing force of the shells for each mussel host as described in section 2.5. (not done for the first month).

## 2.8. Statistical analysis

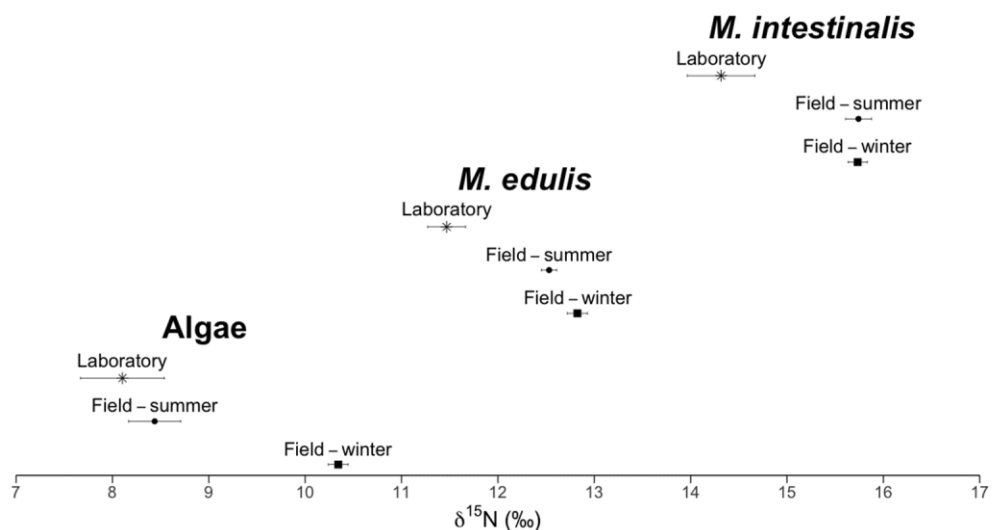
All statistical calculations were performed with [R], (R Core Team (2015). R: A language and environment for statistical computing. R foundation for Statistical Computing, Vienna, Austria. URL <http://www.R-project.org/>). Prevalence of parasites is defined as the percentage of infested host individuals within the total sample  $\pm$  SE and the mean intensity is defined as the mean number of parasite individuals living in one host individual within the total sample, while  $n$  of uninfested hosts is excluded  $\pm$  SE. We analyzed the differences of  $\delta^{15}\text{N}$  values between mussels and parasites as well as between mussels and food sources by using the Kruskal-Wallis test. For analyzing and comparing the metabolic profiles between uninfected and infected mussels the software ‘Metaboanalyst 3.0 (Xia & Wishart, 2016) was used. A generalized log-transformation was applied to the metabolite concentrations to reduce the variance across samples prior to – univariate and multivariate statistics. Unsupervised principle component analysis (PCA) was applied between groups to identify potential outliers. A sparse partial least squares classification for high dimensional metabolomic data (sPLS-DA) was used to find differences between the metabolomic profiles of parasitized and non-parasitized mussels and to identify the most relevant metabolites responsible for significant differences. The quality of the spectra of one adductor muscle sample of the infected mussel group did not allow an analysis of metabolites and was

omitted. In addition, four mussels of the infected mussel group were excluded from the analyses where the infection intensity was low (one or two parasites per mussel). To find significant differences of certain traits (condition, growth and shell cracking force) between the parasitized and the non-parasitized mussel group we used optimized GLMs by using each mussel as statistical unit. The used models are given in detail within the results part.

### 3. Results

#### 3.1. Stable isotope analyses

The parasite intensity of the experimentally infected mussels was  $16 \pm 2$  parasites infected host<sup>-1</sup> with a mean shell length of 48 mm.  $\delta^{15}\text{N}$  values of mussel food sources (i.e. suspended particulate organic matter) were the lowest and ranged from 8.1 to 10.3 ‰. C/N ratios of suspended particulate organic matter ranged from 6.9 (summer field experiment) to 10.3 (winter field experiment), with SPOM provided during the *in vitro* experiment having an intermediate C/N ratio of 7.5. The  $\delta^{15}\text{N}$  values of SPOM were followed by the mussel  $\delta^{15}\text{N}$  values (ranging from 11.5 to 12.8 ‰) and the parasite  $\delta^{15}\text{N}$  values (from 14.3 to 15.7 ‰) that were highest, Fig. Ch.I.1.



**Figure Ch.I.1.:** Mean  $\delta^{15}\text{N}$  values of mussel food sources (Algae), mussels (*M. edulis*) and parasites, (*M. intestinalis*).

Mussels and parasites from the field had very similar  $\delta^{15}\text{N}$  values within groups independent from time of the year whereas suspended particulate organic matter had slightly higher  $\delta^{15}\text{N}$  values (1.9 ‰) in winter than in summer, Fig. Ch.I.1. The difference of  $\delta^{15}\text{N}$  values ( $3.0 \pm 0.1$  ‰) between mussels and parasites was constant in the three experiments (Kruskal-Wallis test,  $H = 3.165$ ,  $df = 2$ ,  $p = 0.206$ ). The difference of  $\delta^{15}\text{N}$  values between mussel food sources and mussels was more variable (from 2.6 to 4.1 ‰), (Kruskal-Wallis test,  $H = 7.467$ ,  $df = 2$ ,  $p = 0.024$ ) with a lower difference observed in the laboratory and the winter field experiment compared to the summer field experiment.

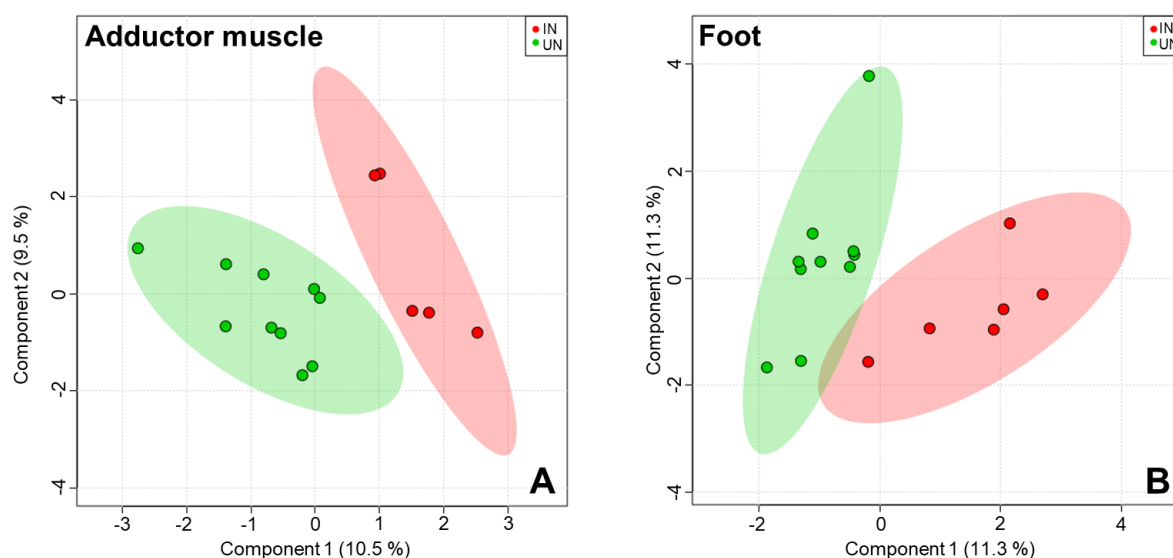
### 3.2. NMR analysis

All mussels used for untargeted NMR based metabolic profiling had a similar size range that differed not significantly between the parasitized and non-parasitized groups (mean shell length of 4.3 cm, GLM, Gaussian,  $\chi^2 = 0.036$ ,  $p = 0.6$ ). The mean intensity of uninfected mussels was significantly lower, (1.0 infected host<sup>-1</sup>,  $n = 3$  out of 10) than in infected mussels ( $22 \pm 2.1$  infected host<sup>-1</sup>,  $n = 6$  out of 6, GLM, negative binomial,  $LR\chi^2 = 232.95$ ,  $p < 0.001$ ). We identified 28 distinct metabolites within the mussels' tissues (posterior adductor muscle, foot, gills and mantle, see Tab. Ch.I.1.).

**Table Ch.I.1.:** List of compounds (28) identified in the <sup>1</sup>H-NMR spectrum of the posterior adductor muscle, foot, gills and mantle tissue extracted of *M. edulis* infected with *M. intestinalis* and non-infected blue mussels.

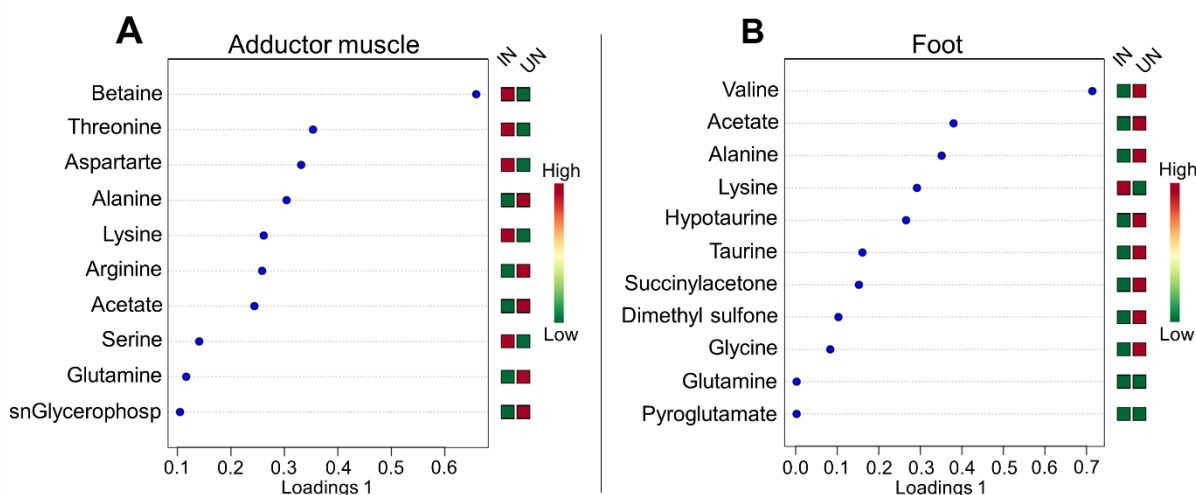
<b>Metabolite</b>	
<u>Amino acids</u>	<u>Energy metabolism</u>
Alanine	Acetylcholine
Arginine	Adenosine triphosphate
Aspartate	Lactate
Glutamate	
Glutamine	<u>Krebs cycle intermediates</u>
Glycine	Succinate
Isoleucine	
Leucine	<u>Metabolism of organic osmolytes</u>
Lysine	Dimethyl sulfone
Methionine	
Serine	<u>Organic osmolytes</u>
Threonine	Betaine
Tyrosine	Glycerophosphocholine
Valine	Homarine
	Hypotaurine
<u>Carbohydrate metabolism</u>	Pyroglutamate
Acetate	Taurine
Succinylacetone	Trimethylamine-N-oxide

Neither the univariate statistics nor the multivariate statistics revealed significant differences between individual metabolites from the gills and mantle tissue of the two mussel groups. Univariate statistics revealed no significant differences between individual metabolites from adductor and foot muscle tissue of the two groups. The principal component analysis (PCA) showed no differences between the normalized concentrations between both groups and no outliers were detected within samples and tissues. However, the sparse partial least squares classification for high dimensional metabolomic data (sPLS-DA) of the adductor muscle showed a clear separation of the metabolomic profiles between uninfected and infected mussels of both tissues. Figure Ch.I.2. shows the scores of the first and second component of the sPLS-DA in a score plot. Both groups are significantly separated indicated by the ellipses representing their 95 % confidence interval, Fig. Ch.I.2.



**Figure Ch.I.2.:** Sparse partial least squares classification for high dimensional metabolomic data (sPLS-DA) of both mussel groups (green uninfected [UN] and red infected [IN]) for the adductor muscle (A) and the foot tissue (B).

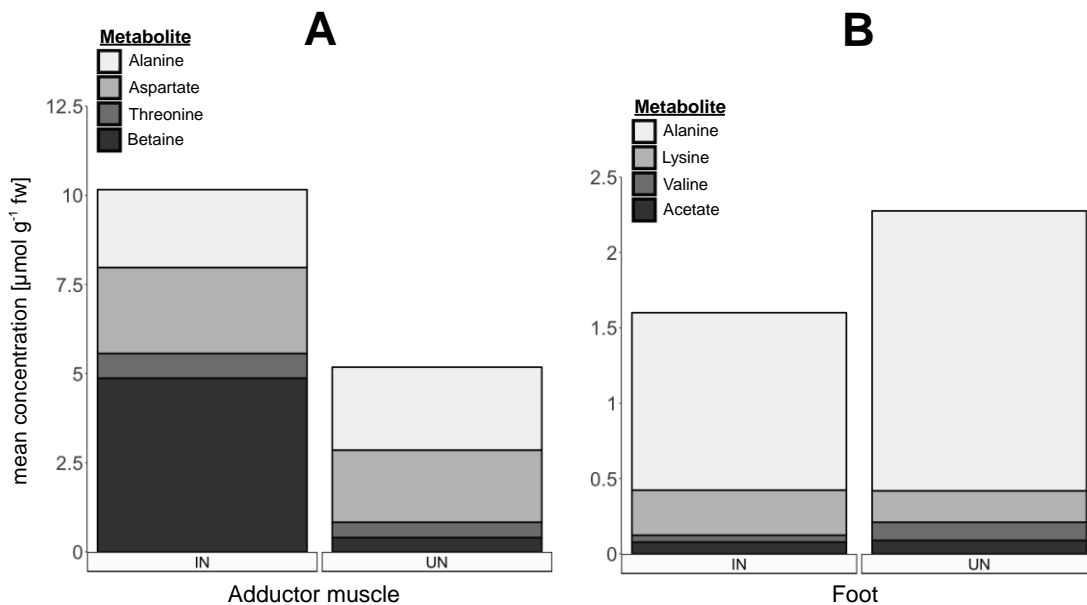
The variable importance in projection (VIP) scores shows the metabolites that contribute significantly to the separation of samples from the different mussel groups (infected and uninfected mussels) within the sPLS-DA model for the adductor muscle (Fig. Ch.I.3., A) and for the foot tissue (Fig. Ch.I.3., B).



**Figure Ch.I.3.:** Loadings plot showing the variables selected by the sPLS-DA model of both mussel groups (infected [IN] and uninfected [UN]) for the adductor muscle (A) and the foot tissue (B). The metabolites are ranked by the absolute values of their loadings.

According to the sPLS-DA the osmolyte betaine and the  $\alpha$ -amino acids threonine and aspartate were the most responsible for the significant separation between the two mussel groups in the adductor muscle,

Fig. Ch.I.3./Ch.I.4., A. Compared to non-parasitized mussels these three metabolites were increased in concentration in parasitized mussels, Fig. Ch.I.3./Ch.I.4., A. Alanine in turn was decreased in the adductor muscle of parasitized mussels compared to non-parasitized mussels.



**Figure Ch.I.4.:** Mean concentrations of metabolites that were different in the fresh weight (fw) of the adductor muscle (A) and the foot tissue (B) between infected and uninfected mussels. Note the increase of betaine in the adductor muscle and the decrease of alanine in the foot tissue of infected mussels, whereas the  $\alpha$ -amino acids aspartate, threonine and lysine are increased.

In addition, the sPLS-DA analysis of the foot tissue samples revealed also a significant separation between both groups, Fig. Ch.I.2., B. In the foot tissue, alanine again and the metabolites acetate and valine were decreased within parasitized mussels compared to non-parasitized mussels, whereas the  $\alpha$ -amino acid lysine in turn was elevated within the infected group, Fig. Ch.I.3./Ch.I.4., B.

### 3.3. Mussel condition and growth experiments – field and laboratory

#### 3.3.1. Parasite intensity and prevalence of the field experiment

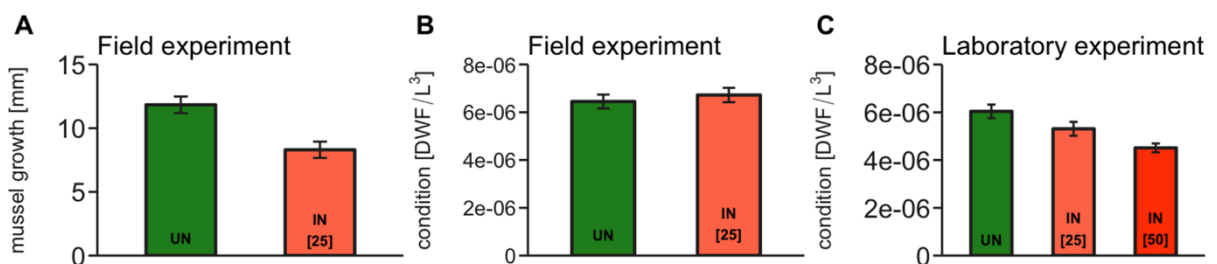
After one year, 51 out of the 72 cages could be retrieved from the field, (26 infected mussels and 25 uninfected mussels). Two infected mussels died during the one-year survey. None of the uninfected mussels died. Both mussel groups were parasitized after the experiment. In the following, we term initially non-parasitized mussels as ‘uninfected’ mussels and experimentally infected mussels as ‘infected’ mussels. The prevalence of infected mussels was 100 % and the prevalence of uninfected mussels was 96 % after the one-year field experiment. The infection intensities were significantly different between the two mussel groups (parasite intensity, GLM, negative binomial,  $LR\chi^2 = 16.705$ ,  $p < 0.001$ ), with infected mussels showing a higher intensity of  $12 \pm 1.2$  parasites while uninfected mussels collected  $6.2 \pm 0.89$  new parasites per host<sup>-1</sup> during the field exposure.

### 3.3.2. Parasite intensity and prevalence of the laboratory experiment

At the end of the experiment, the parasite intensity was significantly different between the three different infection groups (uninfected mussels, moderately infected mussels and highly infected mussels). The mean intensity increased with the experimental infection load (parasite intensity, GLM, negative binomial  $LR\chi^2 = 337.52$   $p < 0.001$ ). Mean intensity in mussels was  $1.6 \pm 0.3$  parasites infected host<sup>-1</sup> for uninfected mussels,  $19 \pm 0.82$  parasites infected host<sup>-1</sup> for moderately infected mussels and  $28 \pm 2.5$  parasites infected host<sup>-1</sup> for highly infected mussels.

### 3.3.3. Mussel condition and growth

The condition of mussels was decreasing with the amount of parasites in all the experimental setups and for the wild mussels out of the field survey. This trend was however not significant for all the experiments. In the field infection experiment, the condition index of mussels was significantly decreased for mussels with a high amount of parasites, with females showing a higher impact than males. The full model with females, males and infection status as fixed effects had a log-likelihood of 594.43 (df = 9). We optimized this model by stepwise removal of interaction terms that did not explain a significant proportion of the variation. The optimized model had a log-likelihood of 592.91 (df = 4) and contained females and infection status as fixed effects (GLM, mussel condition ~ females + infection status). The infection status had a marginally significant effect on mussel condition, Fig. Ch.I.5. (B) and the amount of females had a significant negative effect on mussel condition, (condition index ~ females, GLM, Gaussian,  $LR\chi^2 = 5.7$   $p = 0.017$ ) and (condition index ~ infection status, GLM, Gaussian,  $LR\chi^2 = 2.923$   $p = 0.087$ ).



**Figure Ch.5.:** Mussel growth (A) and condition (B) of the field experiment mussels and mussel condition of the laboratory experiment mussels (C). Shown are the mean ( $\pm$  SE) for uninfected mussels (green) and experimentally infected mussels (red color grades). The number of copepods used for infection is given by the bracketed number within the red bars.

For the laboratory experiment the full model (with females, males and infection status as fixed effects) and moderately and highly infected mussels for condition analysis had a log-likelihood of 280.042 (df = 9). As this model showed no significance, we optimized the full model by stepwise removing interaction terms that did not explain a significant proportion of the variation. The optimized model contained only infection status as fixed effects and had a log-likelihood of 277.465 (df = 3),



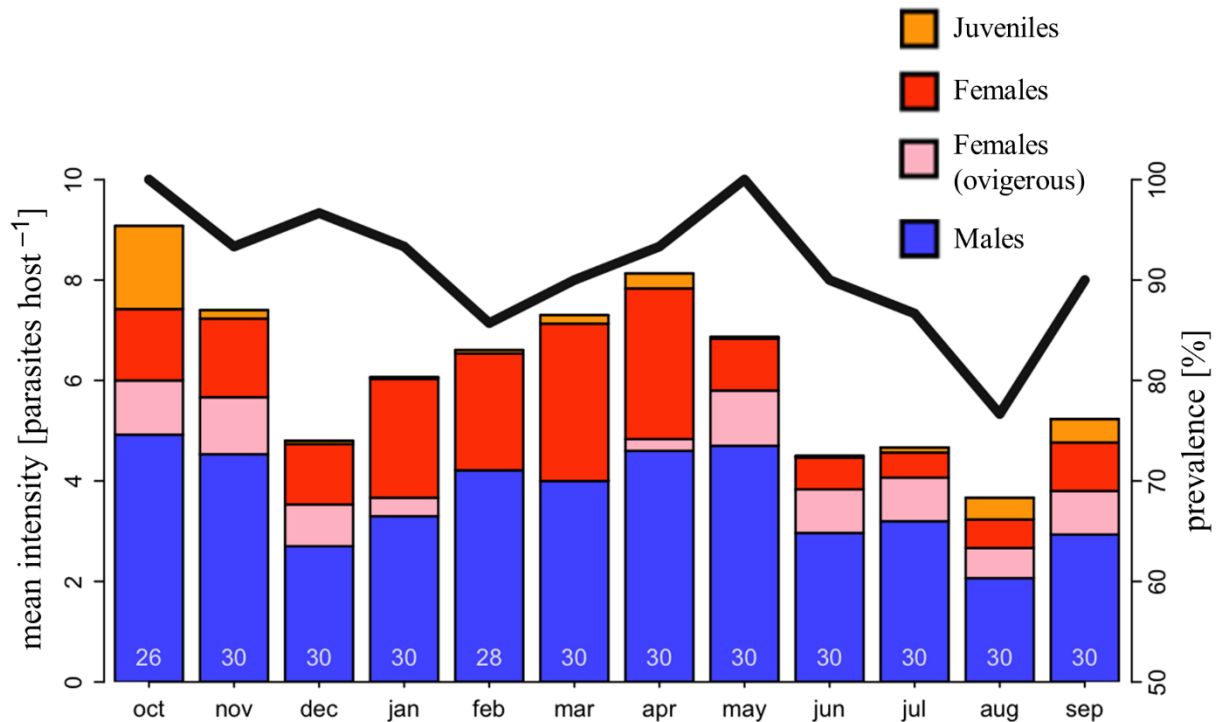
condition, GLM, Gaussian,  $LR\chi^2 = 4.879$   $p = 0.027$ , Fig. Ch.I.5. (C). Additionally, this relationship was tested with the optimized model for comparison with the uninfected mussel group. The condition was significantly different between the highly infected mussel group and the uninfected mussel group, (condition, GLM, Gaussian,  $LR\chi^2 = 18.305$   $p < 0.001$ ). Condition was only marginally significantly different between the uninfected mussel group and the moderately infected mussel group (condition, GLM, Gaussian,  $LR\chi^2 = 3.203$   $p = 0.074$ ), Fig. Ch.I.5. (C).

The mussel growth experiment in the field revealed that uninfected mussels grew faster than infected mussels after one year, Fig. Ch.I.5. (A), while the relationship between individual parasite load and growth showed a similar negative trend in both groups. The full model with females, males and infection status as fixed effects had a log-likelihood of -122.974 (df = 9). We optimized the full model by stepwise removing interaction terms that did not explain a significant proportion of the variation. The optimized model with a log-likelihood of -123.507 (df = 4) only contained females and infection status as fixed effects, (mussel growth ~ females, GLM, Gaussian,  $LR\chi^2 = 4.066$   $p = 0.044$ ; mussel growth ~ infection status, GLM, Gaussian,  $LR\chi^2 = 6.686$   $p < 0.01$ ). The experimental infection led to an overall higher parasite load and a higher reduction in growth indicating that the negative effects on growth occurred later in uninfected mussels and resulted from new infections collected in the field, whereas experimentally infected mussels suffered from infection over the whole experimental period.

### 3.4. Field survey

Throughout the year, the mean parasite intensity was  $6.7 \pm 0.26$  parasites per infected host with a prevalence of 91 %. The uninfected mussels from the field experiment collected a similar parasite load in one year ( $6.2 \pm 0.89$ ), while the experimental infections over all experiments lead to a higher parasite infection intensity (see above). Nevertheless, experimental infection intensities were well within the range observed in the wild and even the maximum infection intensities of parasites per host were comparable (35 adults in the field and 39 adult parasites in the experimental infections).

Figure Ch.I.6. shows the seasonal occurrence of *M. intestinalis* from October 2014 to September 2015 with the mean intensity of males, females / ovigerous females and juveniles as well as the parasite prevalence. Prevalence correlated significantly with intensity throughout the year (pearson=0.62) and both measures showed two peaks one in spring and one in autumn.



**Figure Ch.I.6.:** Mean parasite intensity (bars corresponding to left y-axis) and prevalence (solid line corresponding to second y-axis) of *M. intestinalis* observed in a wild mussel bed. The field survey was conducted for one year from October 2014 to September 2015. Bar shading represents different parasite classes and the number within the bars represent the number of mussels collected (>2 cm shell length).

Males were always more numerous than females resulting in a male-biased sex-ratio. Reproductive females could be found throughout most of the year (only in February and March eggs were lacking), but high numbers of juveniles could only be found in summer and in autumn after periods of comparatively low parasite prevalence. The amount of parasites significantly increased with mussel size with bigger mussels generally have higher parasite counts (total parasite count ~ mussel length, GLM, negative binomial,  $LR\chi^2 = 42.184$   $p < 0.001$  and total parasite count ~ month, GLM, negative binomial,  $LR\chi^2 = 50.426$   $p < 0.001$ ).

The condition index of wild mussels was not significantly decreased due to a high amount of parasites but was significantly different for different seasonal times of the year. The in Tab. Ch.I.2. given model for the condition index had a log-likelihood of 4193.369 (df = 49). After stepwise optimization the log-likelihood was 4175.525 (df = 14).

**Table Ch.I.2.:** Condition index of mussels out of the field survey.

Effect Response	condition index <i>field survey</i>	condition index <i>field survey</i> (after model optimization)
Model: GLM(CI ~ month * females * males) distribution: <i>Gaussian</i>		
	LR $\chi^2$	LR $\chi^2$
Month (MO)	32.478 <sub>df = 11</sub> ***	119.324 <sub>df = 11</sub> ***
Females (F)	0.103 <sub>df = 1</sub>	2.216 <sub>df = 1</sub>
Males (MA)	1.676 <sub>df = 1</sub>	nf
(MO) x (F)	7.453 <sub>df = 11</sub>	nf
(MO) x (MA)	7.018 <sub>df = 11</sub>	nf
(F) x (MA)	4.002 <sub>df = 1</sub> *	nf
(MO) x (F) x (MA)	7.399 <sub>df = 11</sub>	nf

The shell strength of mussels out of the field survey and out of the field experiment was not significantly decreased due to parasite infection, Tab. Ch.I.3. For the field survey the main effects on mussel shell strength were shell length and the seasonal timing.

**Table Ch.I.3.:** Shell strength of mussels out of the field survey and the field experiment, (after model optimization).

Effect Response	shell strength <i>field survey</i>	shell strength <i>field experiment</i>
<u>Model field experiment:</u> GLM(shell strength ~ shell length + month * females * males) distribution: <i>Gaussian</i>		
<u>Model field survey:</u> GLM(shell strength ~ shell length + females * males * infection) distribution: <i>Gaussian</i>		
	LR $\chi^2$	LR $\chi^2$
Shell length (SL)	80.073 <sub>df = 1</sub> ***	nf
Month (MO)	95.124 <sub>df = 10</sub> ***	nf
Females (F)	nf	nf
Males (MA)	nf	3.787 <sub>df = 1</sub> .
Infection status (I)	nf	nf

#### 4. Discussion

In the Wadden Sea, epibenthic bivalve populations such as mixed beds of blue mussels *Mytilus edulis* and oysters represent hot spots of biodiversity (Buschbaum *et al.*, 2009). Hence, impacts of non-native parasites on those ecosystem engineers can affect the occurrence and diversity of a wide variety of associated species. Thus, showing the importance to explore the direct effects of invasive parasites on native mussels with which they live in close contact. Generally, the impact of invasive parasites on the host is diverse with several coinciding direct effects affecting the host in multiple ways. In this study, we explored the direct effects of the invasive parasite *Mytilicola intestinalis* on blue mussels *Mytilus edulis* with an interdisciplinary approach. We determined the nitrogen isotopic composition of parasites, hosts and hosts' food sources. Afterwards, we studied how the growth rate as well as the

condition of mussels varies depending on the infection rate with the parasite and compared the metabolomic profiles between non-parasitized and parasitized mussels. Furthermore, we investigated the intensity and prevalence of the parasite within a mussel bed at the Sylt-Rømø Bight, North Sea to assess the natural burden of *M. edulis* and thus could extrapolate the direct effects of *M. intestinalis* to wild mussels. Our results show that *M. intestinalis* is feeding on the hosts' tissue and alters the metabolomic profile of its host resulting in an altered energy budget and reduced mussel growth. Mussels out of the laboratory experiment showed a decreased condition due to an increased parasite infection. Whereas mussel condition was generally decreased with the number of parasites in all the experiments (laboratory and field experiment) as well as in the field survey, the results of the condition measurements for mussels out of the field experiment showed no significant difference in mussel condition between the two infection statuses (non-parasitized and parasitized mussels). The shell length of mussels is generally a robust parameter that can be used to reveal differences in certain traits of mussels like age and can also be used within condition measurements. The static ratio (with cubed shell length as a component) as indicator for body condition could however not have been suitable enough for comparing body condition between mussel groups with different shell lengths within our experiments. We therefore concentrate on growth as a proxy for condition which was significantly decreased for infected mussels within the field experiment. After one year in the field, experimentally infected mussels grew significantly less than uninfected mussels. In contrast to the laboratory and field experiment, we could not find a significantly decreased condition due to a high parasite intensity within wild mussels. This might suggest that the static condition measurement of the ratio of dry flesh weight to cubed shell length may not be sensitive enough to detect variations in the condition of infected mussels in the wild. It may also be possible that the condition of mussels in the wild is not impaired by pure parasite infection. Further factors may be necessary to induce a loss of condition like adverse environmental conditions for the mussels or additional stressors, (maintenance under laboratory conditions). Furthermore, the parasite incidence of the surveyed wild mussel bed revealed that during phases of potential high energy demand for mussels (spawning period in summer) the reproduction phase of the parasite (amount of eggs) was also high, although the overall parasite intensity was low. The reduced number of parasites in summer may compensate the negative direct effects on infected mussels additionally demanding energy for spawning.

*Mytilicola intestinalis* is an intensively studied invasive parasite with gross discrepancies concerning its effect on the host. Very early studies confirmed its pest status (Cole & Savage, 1951; Meyer & Mann, 1950) whereas later studies did not report any detrimental effect on the host (Davey, 1989; Dethlefsen, 1975). An understanding of the mode of feeding of *M. intestinalis* and its true diet is an important information to term it as a true parasite. As inhabiting the intestine, several food sources are principal available by the parasite. These are undigested phytoplankton as the major food source of blue mussels, breakdown products of the host, or tissue of the gut epithelium (Gresty & Quarmby, 1991). To

distinguish between ingested and assimilated food and thus to reveal trophic relations between *M. edulis* and *M. intestinalis*, stable isotopic data are necessary (Pinnegar & Polunin, 2000). Especially using stable isotopes of nitrogen can reveal true trophic relationships between species (DeNiro & Epstein, 1981). Isotopic compositions confirm that *M. intestinalis* is a real parasite. The much higher  $\delta^{15}\text{N}$  values of *M. intestinalis* than of *M. edulis* clearly confirm that this parasite is not competing with its host for food, but that it is directly feeding on host tissue. The differences of  $\delta^{15}\text{N}$  values between *M. intestinalis* and *M. edulis*, ( $3.0 \pm 0.1$  ‰) are indeed very well in the range of the trophic fractionation factors generally measured between two trophic levels within aquatic invertebrates:  $3.23 \pm 0.41$  ‰ (Vander Zanden & Rasmussen, 2001); 3.4 ‰ (Minagawa & Wada, 1984; Post, 2002). This is in accordance with a study of Gresty and Quarmby, (1991) who showed higher  $\delta^{15}\text{N}$  values for *M. intestinalis* but they debated that this might also result from a faster accumulation of  $^{15}\text{N}$  in the parasite than in the mussel, (Gresty & Quarmby, 1991). With our experiments we could clearly show, that *M. intestinalis* is feeding on the host tissue. The different C/N ratios of SPOM provided to the mussels did not influence the differences of  $\delta^{15}\text{N}$  values between hosts and parasites. Therefore, the parasitic behavior of *M. intestinalis* remained the same when the quality of the hosts' food resource was changing. The differences of  $\delta^{15}\text{N}$  values between *M. edulis* and suspended particulate organic matter (2.6 to 4.1 ‰) are in the ranges of the trophic fractionation factors observed between primary consumers and their food sources,  $2.52 \pm 2.50$  ‰ (Vander Zanden & Rasmussen, 2001) indicating that mussels were relying on the suspended particulate organic matter. If *M. intestinalis* would have relied as well on *M. edulis* food sources (i.e. suspended particulate organic matter) then the parasite would have had similar  $\delta^{15}\text{N}$  values as had *M. edulis*, which was not the case. Similar differences of  $\delta^{15}\text{N}$  values were measured between the parasites and the hosts within the two seasons, suggesting that *M. intestinalis* behaves as a parasite all year long. This evidences that this parasite relies on host tissues and very secondly on host gut contents. Additionally, the movements of *Mytilicola* can cause damage on the hosts intestinal wall (Moore *et al.*, 1978). The more the parasite moves within the gut, the higher the mechanical harm is. As especially adult females need to change their position within the intestine of the mussels to release eggs into the sea (Davey & Gee, 1988; Grainger, 1951), it is likely that females have a large impact on the host. Both processes (i.e. eating host tissues and causing lesions on the intestinal wall) are affecting the host with possible consequences for the hosts' energy metabolism.

#### Untargeted metabolic profiling based on $^1\text{H}$ -NMR spectroscopy

The results of the stable isotope analyses demonstrated that *M. intestinalis* is dependent on the hosts' resources to sustain and reproduce. In accordance, the  $^1\text{H}$ -NMR spectra from the adductor and foot muscle revealed a significant separation in the PLS of the metabolomic profiles between parasitized and non-parasitized mussels. By infecting mussels, *M. intestinalis* does affect the metabolic homeostasis of *M. edulis* from which it nutritionally benefits. This may indicate that the parasite is dependent on the metabolites of the mussel (Sobecka, 2012). However, not all of the different mussel tissues that we

investigated were similarly affected. The mantle and gills of *M. edulis* revealed no significant separation of the metabolic profiles between the two mussel groups. Both organs generally provide a large surface able to absorb nutrients that can be incorporated into the mussels' tissues and further processed via metabolic pathways. Especially the gills are responsible for the first uptake of amino acids essential for viable organ functions of organisms (Siebers & Winkler, 1984; Wu, 2009). One main function of the mussel shell for example is the protection against predators. The shell of *Mytilus* is the first line of defense against predators (Freeman, 2007; Leonard *et al.*, 1999) and pathogens (Canesi *et al.*, 2002). The mantle tissue of *M. edulis* is mainly responsible for secreting the shell via biomineralization processes (Waller, 1980). Preserving the viability of the mantle is thus one of the main life-sustaining processes within *M. edulis*. Our results show that the nutrient supply of mantle tissue was not affected by parasite infection or could be compensated by delivering metabolites out of other tissues. Overall, we could show that the mantle and gills of *M. edulis* are not directly affected by *Mytilicola* infection.

However, the adductor and food muscle showed a good separation in the first two components of the PLS-DA between the metabolic profiles of parasitized and non-parasitized mussels. This separation is mainly generated from differences in amino acids between both mussel groups. The foot secretes byssus threads enabling the mussel to stay attached to the ground. Hence, mussels can keep their position and protect against predators (Farrell & Crowe, 2007). Former studies showed that the byssus of parasitized mussels might be weakened by *M. intestinalis* infection (Brienne, 1964). As amino acids are a component of byssus threads, the altered metabolic profile within the foot of parasitized mussels may indicate a change within the amino acid composition of the byssus threads and thereby may indicate a weakened strength of byssus (Lucas *et al.*, 2002).

The separation in the first two components of the PLS-DA of the posterior adductor muscle of *M. edulis* is generated by differences in betaine and amino acids between the two mussel groups. Betaine, (belonging to the osmoregulatory chain) concentration was elevated in the adductor muscle of parasitized mussels. *M. edulis* is an active osmoconformer and needs to regulate its osmotic homeostasis, (Lange, 1963). *M. intestinalis* might uptake ions from its host for own metabolic processes and thus, may disturb the adjustment of ion concentration within *M. edulis*. When being infected with *M. intestinalis* maybe more betaine has to be mobilized in the adductor muscle by the host. Furthermore, the adductor muscle of *M. edulis* is permanently active which may require a continuous energy flow towards this organ. By contracting and relaxing the muscle, *M. edulis* can keep their shells closed or open for filtration processes. The physiological regulation of the degree of valve-opening can help mussels to save energy through reduced metabolism (Tang & Riisgård, 2016) and the adductor muscle is further important within the defense against predators (Hancock, 1965).

The observed differences of the metabolomic profile between parasitized and non-parasitized mussels do not reveal the exact mechanisms of the effects of parasitism on the physiological processes of mussels down to the last detail. However, the results of the untargeted NMR based metabolomic profiling showed that mussels that are infected with *M. intestinalis* are directly impacted in their metabolic functioning. Free  $\alpha$ -amino acids are involved in many physiological processes and a shift generally can indicate stress or other disturbances within the organism (Zurbug *et al.*, 1989). Alanine for example is the main end product in the breakdown of glucose within bivalves (Stokes & Awapara, 1968) and its decrease can reveal that the cellular energy budget might be impaired. The increase of lysine and threonine within parasitized mussels can indicate that the protein biosynthesis is affected. Lysine and threonine might be harvested by protein degradation (Deaton *et al.*, 1984) within parasitized mussels that have a higher demand on those amino acids. Altogether, *Mytilicola intestinalis* directly alters the metabolic homeostasis within *M. edulis* thus, may have further implications for the host (reduced fitness, growth and condition of mussels).

Our results of the field growth experiment showed, that experimentally infected mussels grew less than uninfected mussels. Although the effect of parasites on growth of the host can also result in gigantism, (Chapuis, 2009) there are several parasites of *Mytilus edulis* that reduce growth (Bierbaum & Ferson, 1986; Thieltges, 2006). Reduced growth rates of *M. edulis* were attributed to direct tissue lesions by the parasite or to chronic stress induced by the parasite. However, within this study, we found higher  $\delta^{15}\text{N}$  values within *M. intestinalis* and an altered amino acid composition in the tissue of *M. edulis*. Thus, the reduced growth of mussels parasitized with *M. intestinalis* may be particularly a result of direct tissue disruption. Our study shows that the amount of parasites was mainly responsible for a reduced growth rate. Among, females had a higher impact than males. Females might have a higher nutritional demand than males especially when ovigerous. This could explain why males alone did not had such a high effect on mussel growth although numerously predominant. However, not only the energetic suppression by *M. intestinalis* feeding on host tissue could be a reason for reduced growth rates within infected mussels. Mechanical impact of the host's gut by the body of the parasite might further contribute to an impaired nutrition status. Thus, a high amount of parasites may lead to an intestinal compression or might impair the functioning of the mussels' intestine (Gee & Davey, 1986a; Hiepe *et al.*, 2006). Additionally, as *M. intestinalis* is expected to move down the gut during maturing it can cause damage to the gut cells (Davey & Gee, 1988; Hiepe *et al.*, 2006; Moore *et al.*, 1978). The permanent energy demand for repairing the lesions is energetically costly. The infection intensity of experimentally infected mussels (out of the field experiment) was higher (additionally infested with wild parasites) than for uninfected mussels which might have impaired growth in parasitized mussels more strongly than in non-parasitized mussels. This suggests that a high infection intensity with the parasite *M. intestinalis* is energetically demanding and this energy might ultimately lack for growth.

Although, *M. intestinalis* had a direct negative effect on mussel growth, the strength of mussel shells was not significantly affected. This was in accordance with the untargeted NMR analyses that showed no significant separation in the metabolic profiles of the mantle tissues (responsible for biomineralization of the shell) between parasitized and non-parasitized mussels. Hence, we suggest that a reduced shell growth within parasitized mussels is not due to a general metabolic depression. Rather, a higher energy demand together with nitrogen loss within parasitized mussels may be responsible for a reduced shell growth independent of the duration of parasite infestation. The field survey revealed that *M. edulis* is permanently affected by the parasite with variations in parasite intensity. Although the total amount of parasite females was decreased during the main reproductive phase of *M. intestinalis* in summer compared to the residual months, the reproductive phase also coincides with the main spawning period of *M. edulis* in summer. More juvenile parasites might reach maturity from the summer brood, respectively could infect mussels, compared to the autumn brood. This suggests that the main hazard phase for mussels in terms of infecting new hosts is in summer and autumn.

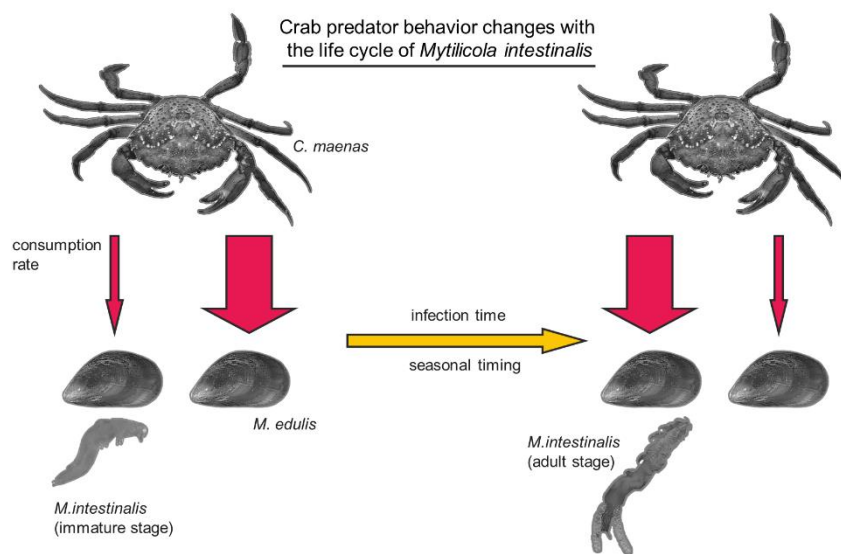
As a conclusion, we could show that *M. intestinalis* clearly exerts several direct effects on *M. edulis*. The stable isotopes of nitrogen revealed that *M. intestinalis* is using host tissue all the year long (in summer and also in winter). We discovered that *M. intestinalis* thereby alters the metabolic profile for parasitized mussels which resulted in reduced mussel growth and condition. However, for wild mussels, the condition was not impaired by *M. intestinalis* showing that the parasite could regulate its direct impacts according to the life cycle of its host or may act as a background stressor with impacts for its host might be more severe when exposed to additional stressors.





## Chapter II

### Infection of blue mussels by parasitic copepods influences crab prey selection





## Infection of blue mussels by parasitic copepods influences crab prey selection

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### Abstract

Parasites can modify the interplay between species and thus affect the outcome of ecological interactions like predation in marine benthic communities. In three laboratory predation experiments we investigated the prey preference of the shore crab *Carcinus maenas* among non-parasitized and parasitized blue mussels *Mytilus edulis* that were experimentally infected by the intestinal parasite copepod *Mytilicola intestinalis*. In no-choice situations, we found no significant differences in consumption rate between parasitized or non-parasitized mussels. However, in two subsequent choice experiments in which non-parasitized and parasitized mussels were simultaneously offered, crabs showed a distinct prey selection depending on the sexual maturity of the parasitic copepods. Crabs significantly preferred non-parasitized prey when infested mussels were infected by immature parasites while a reversed prey choice was observed when the parasites reached sexual maturity. These results show that *M. intestinalis* in mussels can influence the food preference of *C. maenas* according to the developmental stage of the parasite. We concluded that our results highlight the mechanism in which the parasite can optimize its own fitness and transmission by increasing survival rate of immature stages.

### 1. Introduction

Predation and parasitism are fundamental biotic factors that structure coastal benthic communities, (Heithaus *et al.*, 2008; Mouritsen & Poulin, 2010; Thomas *et al.*, 1998). Similar to predators, parasites can control the relative abundance of a host species (Poulin, 1999) and both biotic factors often interact in their ecological functions (Raffel *et al.*, 2008). As parasitism is a ubiquitous component in ecosystems, most of the organisms living in coastal habitats are hosts for parasites (Dobson *et al.*, 2008; Hiepe *et al.*, 2006; Sousa, 1991; Windsor, 1998). Predation on hosts can be modified by parasite infection of prey organisms, especially when predation constitutes an essential transmission pathway for parasite species, (Lafferty, 1999; Moore, 2002; Poulin, 2007). Parasites can make their host more vulnerable for predation and consequently, predators may prey upon a disproportionately large proportion of parasitized, weak or sick prey items (Hudson *et al.*, 1992; Lafferty, 1992; Møller & Nielsen, 2007; Møller *et al.*, 2012; Temple, 1987). An example for such a modification of predator-prey interactions is the intertidal cockle *Austrovenus stutchburyi* in New Zealand that is regularly infested by the trematode *Curtuteria australis*. The trematode encysts the foot of the cockle (Thomas & Poulin, 1998) inhibiting the host's ability to bury in the sediment and subsequently making its host more susceptible to predation by oystercatchers,

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the parasite's definite host (Mouritsen, 2002; Mouritsen & Poulin, 2002). Predation on hosts can be an important factor influencing the life history of a parasite even if it has a direct life cycle (Moore, 2002). This was demonstrated for monogeneans that extend their host range (Strona, 2015). Direct lifecycle parasites can enhance their own transmission by retarding or preventing predation of their host. This opposite behavior pattern of a parasite to that of forcing predation on the host, can lead to an increase of infected host individuals remaining in the population (Packer *et al.*, 2003) and thus makes contact and infection of other potential hosts more likely. Less is known about how cryptic endoparasites with direct life-cycles can influence predator-prey interactions between species of coastal communities although concomitant predation of internal parasitic copepods frequently occurs (Johnson *et al.*, 2010).

In the Wadden Sea, the shore crab *Carcinus maenas* is an important predator for bivalves like the blue mussel *Mytilus edulis* with strong effects on mussel population dynamics (Strasser, 2002) and prey selection is a major forcing process in structuring benthic soft-bottom communities (Reise, 1985; Sih *et al.*, 1985). In principle, crabs show an active prey choice following the optimal foraging theory with highest energy gain at little effort as possible for the crab (Ameyaw-Akumfi & Hughes, 1987; Elner & Hughes, 1978; Mascaro & Seed, 2001; Pulliam, 1974). The preference of crabs can be modified – by organisms associated with its prey. This was for example shown for mussels associated with epibiotic organisms like barnacles (Enderlein *et al.*, 2003; Wahl *et al.*, 1997) with the barnacle shells facilitated handling for the crabs. Furthermore, a change in the crabs' prey preference was demonstrated for infections of prey by polychaete parasites. For example the infection of periwinkles *Littorina littorea* by the shell-boring polychaete *Polidora ciliata* was shown to increase susceptibility of snails to *C. maenas* predation which also lasts for large snail size classes normally being outside of the preferred prey size spectrum of the crabs (Buschbaum *et al.*, 2007). Blue mussels are also infected by different families of parasitic copepods in Europe (Clausiidae, Myicolidae and Sabelliphilidae – (Cheng, 1967; Dethlefsen, 1972; Ho, 1980; Monod & Dollfus, 1932; Raffaele & Monticelli, 1885; Steuer, 1902) and especially *Mytilicola intestinalis* is a well established parasite of *M. edulis* in the south-eastern North Sea (Buck *et al.*, 2005; Caspers, 1939; Elsner *et al.*, 2011; Pogoda *et al.*, 2012; Thieltges *et al.*, 2008; Thieltges *et al.*, 2006). To test whether *M. intestinalis* is able to affect crab prey preference, we explored how *M. intestinalis* influences the predator-prey interaction between *C. maenas* and *M. edulis* in an experimental setup. For the parasite, predation on its host is most detrimental when it happens before the parasite has reproduced. Therefore, we hypothesize that crabs consume blue mussels less frequently when infected with immature stages of *M. intestinalis* and that this pattern may change after reproduction of the copepod parasite.

To test this hypothesis, we experimentally infected mussels for three separate prey choice experiments that investigated the parasite induced effects on crab predation behavior at different times in the course of the developmental cycle of *M. intestinalis* infection. As parasite number and impact on the host can

fluctuate over time, size and age (Davey & Gee, 1976; Dethlefsen, 1972; Hopkins *et al.*, 2013; Paul, 1983; Poulin, 1993; Williams, 1967), we quantified the number of consumed non-infected and infected mussels when parasitized with still immature *M. intestinalis* and contrasted these results to an experiment when parasites achieved sexual maturity. If copepod parasitism can influence crab predation, parasitism can ultimately modify both, mussel host and parasite population dynamics and reveal an often understudied aspect of predator-prey interactions in coastal environments.

## 2. Materials and methods

### 2.1. Origin and treatment of mussels

Blue mussels used as prey for experiments were collected on groins at the west shore of the island of Sylt in the northern Wadden Sea (German Bight, North Sea) near the village of Wenningstedt in May 2015 (54°56.522' N, 8°18.946' E). Prevalence of parasites on such exposed sites is usually very low providing a naturally uninfected stock for our experimental purposes. Accordingly, prevalence of the parasite *M. intestinalis* (defined as the percentage of infested host individuals within the total sample  $\pm$  SE) was 6.7 % with a mean intensity (defined as the mean number of parasite individuals living in one host individual within the total sample, while uninfested hosts are excluded  $\pm$  SE) of  $1.1 \pm 0.07$  infected host<sup>-1</sup> ( $n = 300$ ).

In the laboratory, mussels were separated from each other, rinsed with tap water and all attached epibionts (e.g. barnacles) were removed. Only mussels with a shell length from 2.6 cm to 2.9 cm were used for the experiments because this size class constitutes an optimal diet for shore crabs *C. maenas* with a size of 5.0 cm to 5.9 cm carapace width (Elnor & Hughes, 1978; Enderlein *et al.*, 2003) that we used in our experiments. Uninfested mussels and those that were designated for parasite infection were kept separately in two 28 L (volume of water  $\approx$  15 L) aquaria in a constant-temperature room at 17 °C. Both aquaria had their own independent ambient running natural sea water supply. The water was cleaned before entering the aquaria with a 50  $\mu$ m filter to prevent subsequent cross-infections by spread of small *M. intestinalis* nauplii of a size of 0.2 mm (Caspers, 1939; Gee & Davey, 1986b; Pesta, 1907). Mussels were fed daily with Isochrysis 1800™ (ReedMariculture Inc., U.S.A.) instant algae.

### 2.2. Origin of parasites and infection procedure

After acclimation to lab conditions, half of the mussels were experimentally infected with *M. intestinalis*. This was done by removing embryo sacs of *M. intestinalis* from ripe females extracted *in situ* from the digestive tract of *M. edulis*. These mussels were collected from a littoral mussel bed located at the east side of the island of Sylt in May 2015 (List east; 55°01.331' N, 8°26.456' E) where mussels are heavily infested with *M. intestinalis*. Prevalence at that time was 100 % with a mean intensity of  $6.9 \pm 0.62$  host<sup>-1</sup> ( $n = 30$  mussels).

Embryo sacs were incubated individually in 24 cell culture plates. Wells (3.5 mL) of the culture plates were filled with sterile filtered seawater (0.45 µm) to maximize rearing success as this is inhibited by bacterial pollution (Caspers, 1939). Culture plates were kept at room temperature until naupliar larvae hatched which required one day to several days depending on the developmental stage of the egg-sacs at the time of picking them. When copepods reached the infective first copepodid stage, they were used to infect mussels. With a glass pasteur pipet, sets of 25 copepodids were transferred into 200 mL plastic bottles. Afterwards one mussel was put into each bottle and then covered with filtered seawater. Overnight mussels were allowed to ingest the copepodids. After an exposure time period of at least six hours, it can be expected that more than 75 % of the copepods are ingested by a mussel (Gee & Davey, 1986a). Mussels that should not be infected were treated in the same way but with no copepods in the bottle. Infection procedure was completed at the end of May. After a growth period of two months parasites reached adulthood but were still sexually immature. At this developmental stage of the parasites, we conducted the first predation experiments.

### **2.3. Origin and treatment of predators**

Shore crabs, *Carcinus maenas*, of 5.0 cm to 5.9 cm carapace width were collected by ship based dredging close to the collection site List east and were put individually in 6 L aquaria in the laboratory. To avoid food-based conditioning, mussel tissue from an independent uninfected source from the west southern tip of the island (Hörnum west; 54°45.041' N, 8°16.816' E) was used for initial feeding. There, mussels grow on artificial coastal protection structures (tetrapods) and show a low parasite prevalence of 2 % and mean intensity of 2 host<sup>-1</sup> ( $n = 50$ ). After 24 hours, only crabs that did not consume all the mussel tissue (assuming that these crabs were satiated after feeding) were considered for the experiments. They were then starved for one day to standardize hunger level. Only male crabs were used to prevent potential distortion caused by sexual differences in morphology and predatory behavior, (Crothers, 1967; Elner, 1978; Reise, 1985). For the no-choice experiment, crabs were randomly assigned to groups feeding on uninfected or on infected prey mussels prior to the trials. Feeding groups were matched for coloration to account for potential behavioral differences and prey preference distinctions, (Kaiser *et al.*, 1990; Lewis, 2010) as well as for size (maximum difference for matched crabs within color groups was 0.2 cm) to introduce minimal confounding bias.

### **2.4. Prey choice experiments**

Two types of laboratory prey choice experiments were conducted. In a no-choice experiment only one type of prey (uninfected or infected mussels) was separately offered and in two consecutive choice predation experiments both types of prey were offered simultaneously. Within all experiments shell length of the mussels did not differ for more than 0.2 cm among each mussel pair. All experiments were conducted within 28 L (volume of water  $\approx$  18 L) plastic aquaria with one crab per aquarium. Each

aquarium received its own running seawater supply and was filled with a sediment layer ( $\approx 2$  cm). Sidewalls of the aquaria were covered with tinfoil to prevent interaction among crabs.

At the beginning of the experiments, a single crab was offered two mussels. Within the no-choice experiment, these were either both parasitized or both not parasitized. Within the two choice experiments, one mussel was parasitized and the other was not parasitized. To distinguish between uninfected and infected *M. edulis*, mussels were randomly marked with a dot of red nail polish. The lacquer was sealed with ethyl-2-cyanoacrylate glue. Once a crab had cracked the first mussel, new mussels were offered. After the experiments, mussels were dissected after the last summer experiment and another part after the autumn experiment.

There were 30 individual replicates used in the no-choice experiment (15 replicates feeding on uninfected mussels and 15 replicates feeding on infected mussels). In the first choice experiment, there were 31 individual replicates per treatment and in the second choice experiment 32 individual replicates were used per treatment. Each replicate trial within the experiments lasted for a maximum of eight hours unless a crab made three choices before this time limit. The no-choice and the first choice experiment were both conducted in August 2015, and a second choice experiment was run two month later in October, to test whether prey choice of crabs change with increasing time post-infection of mussels with *M. intestinalis*. To test whether different crab consumption rates between non-infected and infected mussels is depending on a change in the optimal foraging theory; the crabs' handling time was also measured. In the following, the term 'handling time' is used as the time between offering prey to crab until the successful cracking of a mussel and includes recognition time, evaluation time and real handling time as well as the intermission times in between.

## 2.5. Statistical analysis

All statistical calculations were performed within [R], (R Core Team (2015). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL <http://www.R-project.org/>). Prevalence and intensity of field and laboratory infections were analyzed between non-parasitized and parasitized mussels by binomial and Poisson distributed Generalized Linear Models (GLMs). In the no-choice experiment, we analyzed differences in crab consumption of infested and non-infested mussels by using GLMs and differences in handling time of the crabs by linear models using the prey type (infected/uninfected) as predictor. For the combined choice experiments in summer and autumn we used binomial generalized linear mixed models (GLMMs) and analysis of deviance taking each offered mussel as the statistical unit and whether it was consumed as the response variable. The model contained the infection status, season and trial as fixed and crab identity as a random factor. We optimized this model by removing factors that did not have a significant effect on the proportion of explained variation. Similarly, we analyzed handling time except that we used only

handling time of consumed mussels as a Gaussian response variable. Deviance was assumed to be approximately  $\chi^2$  distributed.

### 3. Results

#### 3.1. Parasite infections of experimental mussels

*M. intestinalis* prevalence and intensity in mussels differed significantly between uninfected and experimentally infected individuals (prevalence, GLM, binomial,  $\chi^2 = 74.516$ ,  $p < 0.001$ ; intensity, GLM, Poisson,  $\chi^2 = 452.47$ ,  $p < 0.001$ ). Prevalence of *M. intestinalis* was 7.4 % and intensity was  $1.2 \pm 0.2$  host<sup>-1</sup> for uninfected mussels ( $n=68$ , pooled data of summer and autumn values). For infected mussels prevalence of *M. intestinalis* was 91 % and intensity was  $7.5 \pm 0.99$  host<sup>-1</sup> ( $n = 33$ , pooled data of summer and autumn values), closely resembling the infection rate in *M. edulis* occurring on natural mussel beds (see mussel bed List east [LE], section 2.2.). Prevalence and intensity of *M. intestinalis* in infected mussels decreased between the summer and the autumn experiments. In July intensity of *M. intestinalis* was  $14 \pm 2.1$  host<sup>-1</sup> ( $n = 8$ ) and prevalence was 100 % while in October intensity of *M. intestinalis* was  $5.2 \pm 0.63$  host<sup>-1</sup> and prevalence was 86 % ( $n = 21$ ). For infected mussels the change in parasite intensity between summer and autumn was significant (intensity, GLM, poisson,  $\chi^2 = 63,805$ ,  $p < 0.001$ ). The change of prevalence in mussels between summer and autumn was not significantly different (prevalence, GLM, binomial,  $\chi^2 = 1.76$ ,  $p = 0.185$ ). In uninfected mussels the prevalence and intensity did not change significantly between summer and autumn (prevalence, GLM, binomial,  $\chi^2 = 1.303$ ,  $p = 0.254$ ; intensity, GLM, Poisson,  $\chi^2 = 1.502$ ,  $p = 0.2204$ ). During the experiments in summer, none of the females of *M. intestinalis* had egg-sacs, while 76 % of the female copepods of infected mussels were egg bearing in autumn.

#### 3.2. No-choice experiment in summer

In August 2015, there was no significant difference in the crab consumption between uninfected and infected mussel prey in terms of numbers of mussels consumed (mussel count as a function of infection state, GLM, Poisson,  $\chi^2_{df=1} = 0.672$ ,  $p = 0.412$ ). In total  $1.1 \pm 0.34$  uninfected mussels crab<sup>-1</sup> h<sup>-1</sup> and  $1.4 \pm 0.45$  infected mussels crab<sup>-1</sup> h<sup>-1</sup> were consumed. In addition, the time that the crabs needed to crack the first mussel was not significantly different between uninfected and infected mussel prey (time as a function of infection state, LM, Gaussian,  $\chi^2_{df=1} = 0.1301$ ,  $p = 0.718$ ). To crack uninfected mussels crabs needed  $1.5 \pm 0.2$  hours crab<sup>-1</sup> mussel<sup>-1</sup> and for infected ones  $1.5 \pm 0.37$  hours crab<sup>-1</sup> mussel<sup>-1</sup>.

#### 3.3. Choice experiments

The full model with a binomial error distribution (fixed factors: infection status, season and trial, random factors: crab identity) had a log-likelihood of  $-346.962$  ( $df = 13$ ). We optimized the full model by removing trial as a fixed factor which did not explain a significant proportion of the variation, log-



likelihood  $-348.597$ , ( $df = 11$ ),  $\chi^2_{df=2} = 5.249$ ,  $p = 0.073$ , indicating that the choice preference of each crab did not change significantly in consecutive trials. The optimized model thus contained infection status (presence or absence of *M. intestinalis*) and season as fixed effect and crab identity as random effect (Tab. Ch.II.1.).

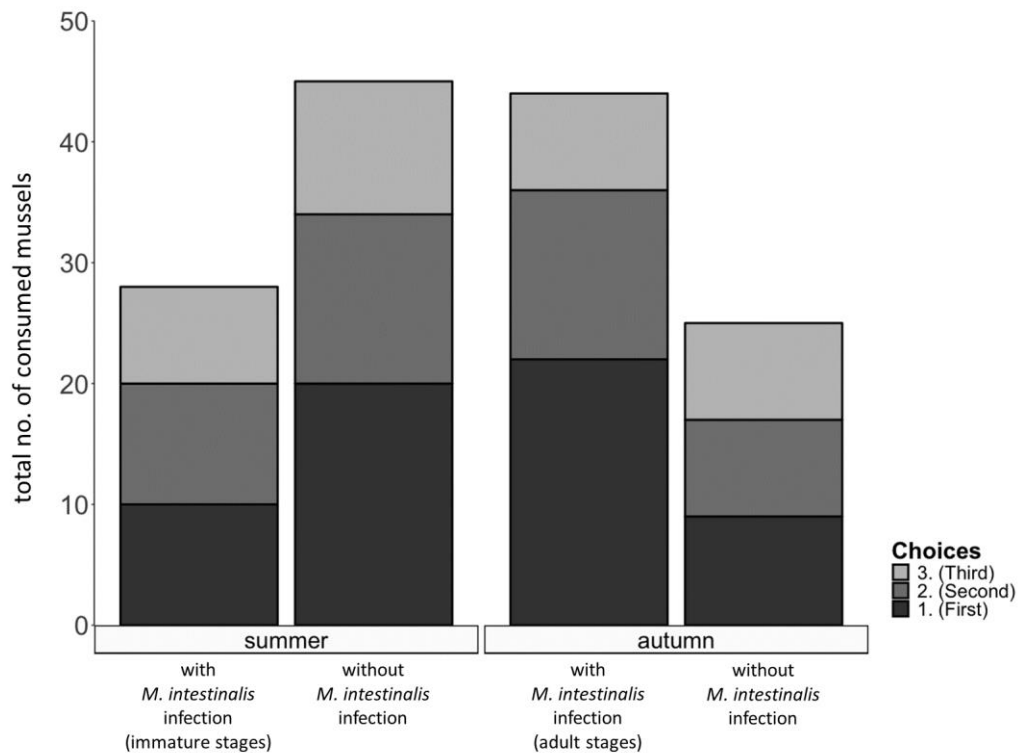
**Table Ch.II.1.:** Optimized GLMM

The optimized model contains mussel chosen or not as response variable, infection status and season as fixed effects and crab identity as random effect, (binomial error distribution).

Significance codes: ‘\*’ refers to  $\leq 0.05$  and is significant ( $\leq 5\%$ ) and ‘\*\*\*’ refers to  $\leq 0.001$  and is very highly significant ( $\leq 0.1\%$ ).

	<b>Chisq</b>	<b>Df</b>	<b>Pr(&gt;Chisq)</b>	<b>Significance-level</b>
<b>(Intercept)</b>	43.592	1	4.045e-11	***
<b>Infestation</b>	6.315	1	0.0119753	*
<b>Season</b>	4.371	1	0.0365580	*
<b>Infestation:Season</b>	11.165	1	0.0008337	***

In general, crabs consumed strongly more mussels of their preferred infection state, leading to an overall higher consumption of parasitized mussels in autumn (Tab. Ch.II.1.). The highly significant interaction term between season and infection status, on the other hand, shows that prey preference changed significantly between seasons (Tab. Ch.II.1.).



**Figure Ch.II.1:** Total number of consumed mussels within the first (dark gray), the second (medium gray) and the third (light gray) choice made by crabs, *C. maenas* when both prey-mussel types were offered simultaneously within one aquarium and one crab each.

In the first choice experiment in summer crabs preferred non-parasitized mussels over those that were infected with immature stages of *M. intestinalis*, Fig. Ch.II.1. In total 45 uninfected and 28 parasitized mussels were eaten. Within the first choice, crabs consumed 10 mussels with *M. intestinalis* infection whereas 20 non-parasitized mussels were consumed. In autumn, by contrast, with *M. intestinalis* individuals reached sexual maturity, crabs preferred parasitized mussels over non-parasitized ones. In total, 25 uninfected and 44 infected mussels were consumed. Crabs consumed 22 mussels with *M. intestinalis* infection whereas only 9 non-parasitized mussels were consumed within the first choice.

The time the crabs needed to crack a mussel was not significantly different between uninfected and infected mussels and also independent from the season (time ~ infection state, LM, Gaussian,  $\chi^2_{df=1} = 0.031, p = 0.86$  and time ~ season, LM, Gaussian,  $\chi^2_{df=1} = 2.566, p = 0.109$ ). The time the crabs needed to crack a mussel was also independent from the experimental setup, meaning independent whether crabs had to choose or not (time ~ setup, LM, gaussian,  $\chi^2_{df=1} = 0.847, p = 0.358$ ).

#### 4. Discussion

Commonly the direction parasites might manipulate their host may depend on the respective lifecycle of the parasite. Hence, lowering the predation risk of the host may be beneficial for direct lifecycle parasites, which is exactly what we observed in our summer experiments where parasites did not yet reach sexual maturity and infected mussels were less often consumed by crabs. The fact that handling time of crabs was not significantly different between non-parasitized and parasitized mussels, suggests that direct parasite manipulation could be responsible for the changed prey selection of crabs instead of side effects caused by energy drain by infection with *M. intestinalis*. Interestingly, the observed pattern was reversed when parasites reached sexual maturity, indicating that manipulation strategies of the parasite might change depending on the parasite's life stage, showing that the resulting direct and indirect effects of parasitism on host population dynamics are not straightforward.

##### **Can immature parasitic copepods avoid being co-consumed with their hosts?**

Our results indicate that a relatively short infection time of two-three months, during which parasites were still sexually immature, was long enough to see an effect of crab predation pressure on mussels. Indeed Campbell (1970) suggested that the larval stages of *M. intestinalis* can even have an effect on mussels. Juvenile stages of *M. intestinalis* may cause more damage to the host than adult stages because they occur not only in the gut (Grainger, 1951) but also in the hepatopancreas and generally mussels infected by only adult stages did not exhibit such high ill-health (Campbell, 1970). Assuming that the juvenile parasites already caused a decrease in condition of their host, the reduced crab predation on parasitized mussels found in our first choice experiment in summer could be explained when weak mussels are less attractive to crabs because providing a low energy content. However, we did not observe differences in handling time between infected and non-infected mussels, suggesting that uninfected mussels were not more attractive to crabs in terms of the optimal diet theorem. Infected mussels were able to resist the crab attacks in the same way as uninfected *M. edulis* did, so we could exclude that side effects of infection occurred. This might suggest that immature stages of *M. intestinalis* took an active part in the shifted crab preference towards uninfected mussel prey.

Generally, predation on immature stages is fatal for the parasite independent whether it has a direct or a complex life cycle and even if the correct consecutive host would eat the intermediate (Dianne *et al.*, 2011; Hafer, 2016; Hafer & Milinski, 2015). Thus, the parasite should prevent predation on the host until it has had time to increase its own transmission and fitness through reproduction. One strategy that could be applied in our experiments is to excrete substances that lower the attractivity of the host, which could be regarded as a direct defense mechanism of *M. intestinalis*. Olfactory cues can be used to indirectly communicate with the external habitat. Hence, *M. intestinalis* might segregate chemical cues to reach other conspecifics. For successful reproduction for example, it is essential that at least one female and one male meet within a single mussel. Thus, chemical cues could ultimately be used to attract

conspecifics to ensure reproduction. Proximately these cues could also be detected by crabs and lower the attractivity of the prey. Gee and Davey (1986a) showed that the presence or absence of a previous infestation of *M. edulis* with *M. intestinalis* did not affect subsequent infestation success. Nevertheless parasitized mussels with low previous parasite loads were infected by a higher parasite number during subsequent infections (Gee & Davey, 1986a), indicating that the copepod parasites are able to recognize the infection status of a mussel before entering it. Many studies confirm, that chemical cues are important in regulating behavior of marine animals and that copepods are able to produce such cues to modify survival, foraging or reproduction or even to find specific hosts (Hay, 2009; Heuschele & Selander, 2014). Chemoreception by *C. maenas* on the other hand is well documented and in the food-seeking behavior of *C. maenas* olfactory ability is very important (Bethe, 1897; Crothers, 1967). It is also conceivable that *M. edulis* releases stress signals caused by being infected with *M. intestinalis* deterring crabs from eating. Regardless of the underlying mechanisms, our results show that direct life cycle parasites such as *M. intestinalis* can relieve predation pressure on their host. The manipulation of predator behavior will thus be beneficial for the parasite because it enhances the survival rate of juveniles until reaching sexual maturity and finally the reproductive success of the parasite.

### **Why does the predator behavior change during the parasite's life cycle?**

The effect of parasites on predation of their host can change during the lifetime of the infecting parasites as age and abundance of the parasite fluctuates over time (Moore, 2002). We detected a much lower intensity of *M. intestinalis* (number of parasites per host) in mussels used in our second experiment. While this is puzzling on first sight, several explanations can account for the reversed crab predator behavior pattern. One reason for this might be, the reduced infection intensity compared to the summer experiment. If the signal for predator manipulation is indeed dose dependent the protection effect that we saw in the first experiment might have been too weak and infected mussels were not rejected by crabs anymore. However, since the parasite's effect did not only disappear but was reversed in our experiments, a loss of signal cannot explain our observed results.

As adult *M. intestinalis* are much bigger than immature stages, the impact of parasites could have been higher in the second experiment even if the intensity was decreased. Additionally, infection time was prolonged and consequently the burden for the mussel host lasted longer than in the first experiment. Thus even if many young parasites might cause more damage to mussels (Campbell, 1970), after a certain time accumulated damage of fewer adult infections will exceed the initial damage. At the start of our second choice experiment, parasitized mussels were already infected with *M. intestinalis* for four months. Hence, even a subtle parasitic impact can accumulate over time (Booth *et al.*, 1993), weakening the anti-predator defense of the host. Furthermore, mature infections could have imposed higher costs for the host in our second experiment, because the generation of progeny of *M. intestinalis* probably equates to the highest energy drain for the host making it most vulnerable to predation. Although some

studies (for instance Dethlefsen, 1975 or Gee *et al.*, 1977) stated that the impact of *M. intestinalis* on its mussel host is small especially at favorable environmental factors and Davey (1989) suggested that *M. intestinalis* is more a harmless commensal to the mussel host (Davey, 1989), a variety of negative effects of *M. intestinalis* infections were also reported such as a reduced filtration rate accompanied by a loss of condition and weakened byssus attachment (Bolster, 1954; Brienne, 1964; Korringa, 1968; Meyer & Mann, 1950; Theisen, 1987). As a consequence of a reduced filtration rate, less food intake, and increased metabolic and respiration rates respectively, an accelerated digestion of albumen was shown (Bayne *et al.*, 1978; Hockley, 1951; Meyer & Mann, 1950). Parasites can also cause retarded spawning (Williams, 1969a). An abnormal coloration of the hepatopancreas and the digestive gland, a reduced growth and a stunted crystal stylus is also documented (Bayne *et al.*, 1978; Bolster, 1954; Campbell, 1970; Caspers, 1939). This could have caused a change in crab behavior because weakened mussels may be less resistant against crab predation or crabs could prevent claw attrition by using weakened mussels for crushing as *C. maenas* is known to select small crushable mussels rather than larger and more profitable mussels (Smallegange & Van Der Meer, 2003). However, similar to the first choice experiment, handling time was also not significantly different for parasitized and non-parasitized mussels in the second experiment, indicating that handling effort was not lower for infected mussels in autumn despite the prolonged infection time of *M. edulis*. If *M. intestinalis* did not weaken the infected mussels, another possibility might be that the parasite tissue constitutes a supplemented food gain for *C. maenas*, which would explain its preference for infected *M. edulis* in October. However, since parasite biomass is obtained from the host (Poulin & George-Nascimento, 2007) higher energy content of infected mussels presumable cannot explain the crabs preference for infected mussels in autumn.

Active manipulation of the parasite on the predation behavior of crabs could also be adaptive if predation on infected hosts is increased as it was in our second choice experiment. Firstly, selective removal of exhausted mussels from the host population at a high rate would increase the likelihood for the parasites' offspring to encounter uninfected hosts in good condition. Reproductive stages of *M. intestinalis* could also exert chemical cues that may condition free living copepod stages to avoid already occupied mussels. Those exerted cues could even stimulate *C. maenas* to prey on infected mussel hosts and underlines the benefit for the parasite when predators consume already infected *M. edulis* after the parasite has reproduced. Such a strategy could also reduce the risk of inbreeding and might increase fitness of offspring that does not have to compete with previous infections. The relationship between previous and new infections might however vary with the background prevalence. In situations with high prevalence and intensities like those that we observed in our study population, competition between consecutive infections might be stronger and consequently alter the selective landscape for infectivity as well as host manipulation. Secondly, crab predation might also increase the hatching success of parasite offspring. We observed high hatching rates in our experiments when egg sacs were kept in clean seawater. If hatching success is reduced in the gut environment of the mussel host, predation might free

larger proportion egg sacs from the gut environment to the water column and result in higher hatching rates.

As a conclusion in both our experiments, active manipulation by *M. intestinalis* could be the reason that led to a changed crab prey selection. The fact that immature stages of parasites prevent predation on the host whereas adult stages evoke predation is an often observed phenomenon in parasites' life cycles. Consequently the way of host manipulation depends on the developmental stage of the parasite and a typical example for this stage-dependent switching is the tapeworm *Schistocephalus solidus* (Hafer & Milinski, 2015; Hammerschmidt *et al.*, 2009). Non-infective parasites prevent predation on the host as long as they need to develop before they are infective to the next host and then switch to an opposite host manipulation strategy and enhance predation (Dianne *et al.*, 2011). Koella *et al.* (2002) for example showed a stage-dependent manipulation of the malaria parasite *Plasmodium gallinaceum* on the mosquito host *Aedes aegypti* that was opposed for two different parasite life stages (Koella *et al.*, 2002). The mosquito *A. aegypti* shows a host-seeking behavior that is continued until a certain threshold for ingested blood is reached; above this threshold the host-seeking behavior is inhibited. The oocysts of *P. gallinaceum* cannot be transmitted and thus the parasite decreases the blood-threshold while sporozoites (that can be transmitted to next host) increases blood-threshold to enhance host-seeking behavior of its mosquito host and thus increase probability to reach next host. Parasites are also able to exert various manipulation strategies. Olfactory protection mechanisms together with parasite-induced aposematism were for example shown to be applied by the parasitic nematode *Heterorhabditis bacteriophora* (Jones *et al.*, 2015). This entomopathogenic nematode causes host death in a short time after entering. When the host is killed by the parasite, it is preserved as the parasite is then able to subsist on the host. By aposematism the parasite protects against predators (Fenton *et al.*, 2011). As a dead and immobilized host is more vulnerable to predation and aposematism alone is not effective especially against nocturnally active predators, the parasite additionally uses odor to protect against predators.

These examples show that parasites can use different host manipulation strategies and can adjust them depending on external circumstances respectively stage of infection. Thus, parasites can temporally optimize their manipulation strategies according to the specific needs of the respective life stage (Dianne *et al.*, 2011; Hammerschmidt *et al.*, 2009; Parker *et al.*, 2009). Such stage-dependent parasite manipulation of hosts, increases transmission success and we observed such a change in parasite manipulation in our choice experiments using a direct lifecycle parasite. While sexually immature stages of *M. intestinalis* were able to prevent predation on their mussel host we could show that predation behavior of crabs was reversed when *M. intestinalis* reached the reproductive stage.

Despite some speculative explanations concerning the underlying effects, our experiments show a clear impact of the parasite *M. intestinalis* on prey selection by shore crabs *C. maenas* on blue mussels, which

results in higher survival of immature stages and better infection conditions for the offspring of the parasites. Whether *M. intestinalis* can actively induce host manipulative mechanisms by chemical cues and/or indirectly for example by weakening the host, cannot completely be answered with our experiments. Future research should analyze the relative contribution of direct behavior manipulation and indirect side effects by combining infections of different parasitic life stages in well-nourished and starved mussels. We hypothesize that if direct manipulation of *M. intestinalis* outweighs the indirect effects, higher consumption rates of parasitized mussels (for sexual mature *M. intestinalis*) should be independent of mussel condition, while potential side effects should be amplified in starved mussels. Furthermore, in nature hosts are often infected with several parasites of various life stages. Hence, the induced changes in hosts can be multidimensional with infective parasites often dominating the behavior manipulation (Hafer, 2016; Hafer & Milinski, 2016a; b; 2015; Haine *et al.*, 2005; Poulin, 2013; Thomas *et al.*, 2010). Our results support the widespread occurrence of complex host behavior switches, which seems not to be constrained to trophically transmitted parasites but also exist in direct life cycle parasites.

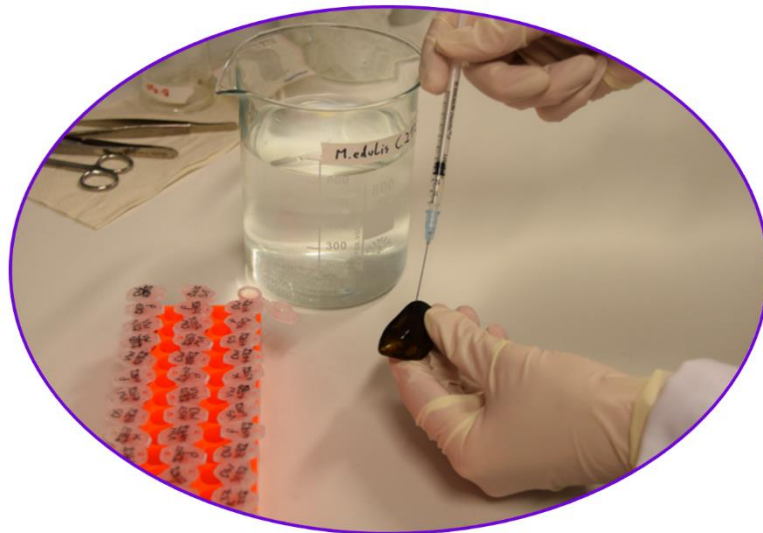






## Chapter III

**Infection by invasive parasites increases susceptibility of native hosts to secondary infection via modulation of cellular immunity**





## Infection by invasive parasites increases susceptibility of native hosts to secondary infection via modulation of cellular immunity

Felicitas Demann<sup>3</sup>, K. Mathias Wegner<sup>3</sup>

### Abstract

Parasite spillover from invasive aliens to native species increases the risk of disease emergence within native biota – either by direct harm to the new host or by indirect effects like increased risks of secondary infection. One example for such a detrimental effect is the parasitic copepod *Mytilicola intestinalis* that infected blue mussels *Mytilus edulis* after being introduced into the North Sea in the early 20<sup>th</sup> century. Since 1949, the parasite was blamed for multiple mass mortalities of infested blue mussels but evidence for a direct causal involvement of *M. intestinalis* remained circumstantial. Here, we now examine the potential effects of primary infections by the invasive parasite on the susceptibility to secondary infections with virulent bacteria (*Vibrio* spp.) in a full factorial infection experiment combining parasite infection (control vs. infected) with different *Vibrio* infection treatments (control, bath challenge, injection) in environmental conditions that either favored the host (ambient temperature) or the bacterium (elevated temperature). The combination of these factors allowed us to differentiate between direct effects on secondary infections by physical wounding of the intestinal parasite (ease of entry) and indirect effects of immune modulation (shifts in immune resource allocation). To do so, we measured the influence of primary and secondary infections on cellular immunity (phagocytosis) and *Vibrio* load in the hemolymph to ultimately correlate these results to host survival. Our results suggest that the rate of secondary *Vibrio*-infection is increased due to lower efficiency of the cellular immune response in mussels infected with *M. intestinalis*. As a consequence, the higher *Vibrio* loads and the failure of clearing *Vibrio* from the hemolymph increased mortality of parasitized mussels. This demonstrates that indirect effects of parasite invasions can outweigh direct effects of the infection highlighting the need for a more integrative approach to understand and predict the consequences of parasite invasions.

### 1. Introduction

Marine bioinvasions are one of the major threats to global biodiversity (Bax *et al.*, 2003; Molnar *et al.*, 2008). Alien species can generally have a variety of effects on native biota (Davis, 2009; Lockwood *et al.*, 2013). Whereas direct impacts are commonly described (Katsanevakis *et al.*, 2014; Parker *et al.*, 1999; Simberloff *et al.*, 2013), indirect effects of invasive species can play an important role and can even exceed the consequences of direct effects (Hatcher *et al.*, 2012; Wootton, 1994). Nevertheless, indirect effects of alien species are often underestimated and especially the impact of parasites and

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diseases as key players in invasions needs to be recognized in this context (Dunn & Hatcher, 2015; Prenter *et al.*, 2004; Tompkins *et al.*, 2011; Torchin *et al.*, 2002). There are different mechanisms how invaders can directly or indirectly affect parasite-host interactions with different ecological and evolutionary implications on natural communities (Goedknecht *et al.*, 2016). The indirect modification of host-parasite interactions by an invasive species can for example result in the emergence of diseases, (Daszak *et al.*, 2000). Consequently the IUCN list of the 100 World's Worst Invasive Alien Species notes parasitic diseases in invasion processes and quotes a quarter of invasive species to be a driver for diseases and to have a negative economic and ecological impact (Lowe *et al.*, 2000). Within these disease-provoking species, eight species are listed to be parasites (Hatcher *et al.*, 2012), where direct effects of infection can intermingle with indirect effects resulting from it. In marine systems, co-introduction of parasites together with invasive species and the subsequent spill over to native species is particularly common and almost a third of parasite co-introductions led to a transfer of parasites from invasive towards native species (Goedknecht *et al.*, 2017; Goedknecht *et al.*, 2016). Among these, nearly half of the new host-parasite systems were associated with mass mortalities of the native host (Goedknecht *et al.*, 2017; Goedknecht *et al.*, 2016). It is however unclear in many cases whether the invading parasite directly cause such mass mortalities or indirectly for example by an increased rate of secondary infections (Ezenwa, 2016). To assess the implications and risks of biological invasions, it is therefore necessary to consider both, direct and indirect parasite-mediated effects.

The copepod *Mytilicola intestinalis* is a good example of an invasive parasite that infected native blue mussels *Mytilus edulis* as a new host after its introduction into the North Sea from the Mediterranean Sea where its principle host is *Mytilus galloprovincialis* (Steuer, 1902). First observations within the North Sea are reported from 1936 in Germany and 1937 in the UK (Caspers, 1939; Cole, 1951). By 1949, not long after the introduction of *M. intestinalis*, the parasite was blamed for blue mussel mass mortalities commonly known as 'red worm pest' that led to the collapse of mussel fisheries in the Netherlands (Korringa, 1951) and Germany (Meyer & Mann, 1950; Meyer-Waarden & Mann, 1954a; b). From the 1960ies to the mid 80ies, mass mortalities of blue mussels infected by *M. intestinalis* also occurred in France (Blateau, 1989; Blateau *et al.*, 1992).

Many adverse direct effects of *M. intestinalis* infections were described that could potentially explain the high mortality rates. Gee and Davey (1986a) for example assumed that at high loads, *M. intestinalis* could cause mussel mortality simply by mechanical blockage of mussel gut (Gee & Davey, 1986a). At cellular level, *M. intestinalis* causes metaplastic changes of cells of the gut epithelium, (Moore *et al.*, 1978) that could further limit gut function (Hiepe *et al.*, 2006). Limited gut function and reduced filtration rates (Meyer & Mann, 1950) coupled to increased metabolic demands originating from the parasite lead to lower mussel condition and could in cases of high parasite load kill the host (Korringa, 1968; Theisen, 1987).

While such negative impacts could indicate a direct implication of this parasite invasion in mortalities, several other studies failed to detect such strong effects (Dethlefsen, 1975) and casted doubt on *M. intestinalis* being the cause for the observed mortalities (Davey, 1989; Dollfus, 1951). These correlative observations, however, also capture the high variation of biotic and abiotic conditions, which can often mask the effect of infection (Gee *et al.*, 1977; Paul, 1983). In the field, mussels are subject to an uncontrolled number of parasite infections and the correlative nature of the results can therefore confound an effect of parasite infection, highlighting the need for experimental studies. In controlled laboratory infections, a negative effect of infection on mussel condition was confirmed but did also not lead to elevated mortality (Feis *et al.*, 2016).

Experimental evidence is therefore lacking for the causal involvement of *M. intestinalis* in mass mortality events and it remains debatable if *M. intestinalis* really caused mortality or just amplified other adverse environmental conditions like increased temperatures or secondary infections with other pathogens (Dollfus, 1951; Lauckner, 1983). High water temperatures can evoke disease associated mass mortalities in blue mussels (Mallet *et al.*, 1990; Myrand & Gaudreault, 1995; Tsuchiya, 1983). Indirect interactive effects of secondary infections and environmental conditions were also observed in *Mytilus galloprovincialis*, where total hemolymph carbohydrate concentrations were reduced when co-infections of *M. intestinalis* and other parasites occurred during hot summer months (Robledo *et al.*, 1995). The constant wounding of gut tissue by *M. intestinalis* infections (Couteaux-Bargeton, 1953; Figueras *et al.*, 1991; Moore *et al.*, 1978; Robledo *et al.*, 1994a; Santarém *et al.*, 1994; Villalba *et al.*, 1997) could additionally provide a constant entry point for secondary infections. And while *Mytilicola* infection did not significantly change hemocyte composition and basic cellular activities in previous studies (Carballal *et al.*, 1998; Moore *et al.*, 1978; Robledo *et al.*, 1994b) immune resources of the host might still be diverted to defense against the parasite (Figueras *et al.*, 1991; Santarém *et al.*, 1994; Villalba *et al.*, 1997) making the host more susceptible to other kinds of infection (e.g. viruses, bacteria). Either way one understudied indirect effect of this parasite invasion might then be an increased rate of secondary infections that could potentially also explain high mortality rates. Since no experimental studies exist that investigate the indirect effect of *M. intestinalis* on secondary infection rates and mortality that arise from those, we now test this hypothesis by exposing mussels that we experimentally infected with *M. intestinalis* to a secondary infection with a pathogenic strain of *Vibrio* spp. By performing the experiment at ambient and raised temperatures, we incorporated adverse environmental conditions and shifted the thermal optimum from mussels with an ambient optimum to bacteria with optima at elevated temperatures. While our focus lay on the secondary infection dynamics (i.e. bacterial load) and its consequence on host survival in general, we also tested immune resource allocation of the host to gain a mechanistic understanding of the effect of parasite and bacterial infections on hemocyte composition and the cellular immune response (phagocytosis). Our full factorial experimental design can therefore reveal the relative importance of the direct effect of an infection to the indirect and

interactive effects resulting from secondary infections and adverse environmental conditions that is essential to gauge and assess the overall impact of parasite invasions.

## 2. Materials and Methods

### 2.1. Origin and treatment of mussels

In March 2015, mussels of 4–6 cm in shell length were collected from the west shore of Sylt, (Wenningstedt; 54°56.522' N, 8°18.946' E) because these mussels provide a stock with very low rates of parasite infections for our experimental purposes. Consequently, the mussels used here showed a prevalence and infection intensity with 6.7 % and  $1.1 \pm 0.07$  parasites, respectively.

After collection, specimens were separated from each other, rinsed with tap water and all attached epibionts (e.g. barnacles) were removed. For the acclimation phase, uninfested mussels were kept in two 28 L (volume of water  $\approx$  15 L) aquaria supplied by fresh seawater flow through.

### 2.2. Experimental treatments

Our experimental design combined *Mytilicola* infection (infected / not infected) and *Vibrio* infections (control / injection / water bath) with the two temperature treatments (ambient and elevated) in a full factorial design, resulting in 12 treatment groups consisting of 10 mussels each.

#### 2.2.1. *M. intestinalis* experimental infection

For *Mytilicola* infections, we collected *M. edulis* from a mussel bed on the East side of Sylt (List east; 55°01.331' N, 8°26.456' E), that is characterized by a high *M. intestinalis* prevalence of 92 % and a mean infection intensity of  $6.7 \pm 0.25$  parasites per host. The digestive tracts of mussels were dissected and the egg sacs of *M. intestinalis* were extracted from ripe females. The egg-sacs were incubated singly in 24 well cell culture dishes. As the developmental process of the embryos is inhibited by bacterial pollution (Caspers, 1939), wells were filled with sterile filtered seawater ( $0.45 \mu\text{m}$  + UV treatment) to maximize rearing success. Egg-sacs were kept at room temperature until the infective first copepodid stage was reached. Twenty-five copepodids were then transferred into 200 mL plastic bottles containing one uninfested mussel each. Mussels were allowed to ingest the copepods over night since an exposure time of six hours should result in more than 75 % of the copepods being ingested (Gee & Davey, 1986a). Control mussels were treated the same way without adding copepodids. Until *Vibrio* infection, mussels were kept singly in a temperature controlled room at 17 °C. Each bottle received its own supply of natural seawater that was filtered at  $50 \mu\text{m}$  and sterilized by UV radiation to prevent subsequent cross-infections by spread of small (0.2 mm) *M. intestinalis* nauplii (Gee & Davey, 1986b; Pesta, 1907). Mussels were fed by adding a constant supply Isochrysis 1800™ (ReedMariculture Inc., U.S.A.) instant algae to the water before being distributed to the bottles.

### 2.2.2. *Vibrio* infection

Once *Mytilicola* reached adulthood in the infected mussels six months after infection, each mussel was transferred to 800 mL beakers filled with sterile filtrated seawater. Treatments were randomly distributed over 8 replicate water baths that were separated by a polystyrene divider. One side of the water bath was filled with ambient (mean ranges within water baths over time, 15.6 °C – 16.7 °C) the other with heated water (mean ranges within water baths over time 17.7 °C – 22.9 °C) to eliminate confounding effects of different treatments being applied in different experimental units (water baths). Each beaker got its own O<sub>2</sub>-supply. Mussels were not fed during the one-week experiment and water was not changed.

A virulent *Vibrio orientalis/tubiashii* strain (D29w, (Lokmer & Wegner, 2015)) was used for the *Vibrio* challenge. Bacteria were grown over night in nutrient medium. Bacterial cells were harvested by centrifugation (3 min at 500 rpm, 6 min at 600 rpm and 3 min at 500 rpm) and resuspended in nutrient medium to obtain a concentration of 10<sup>7</sup> colony forming units (CFU) mL<sup>-1</sup>. We administered *Vibrios* by direct injection into the posterior adductor muscle and by adding bacteria to the beaker. By direct injection of *Vibrios* into the tissue the very first immune barriers of *M. edulis* are overcome and we could analyze if the cellular immune defence against *Vibrio* are impaired in parasitized mussels or at higher temperatures. Bathing challenges on the other hand simulate the natural infection process and tests whether the pathogen load is intensified by an easier entry process resulting from gut wall damage by *M. intestinalis*. To inject *Vibrio*, mussel shells were slightly opened to injecting an inoculum of 0,1 mL into the posterior adductor muscle (10<sup>6</sup> cells per mussel). Since a control injection (for example with seawater) or withdrawal of hemolymph usually does not lead to significantly increased mortalities, (Gauthier-Clerc *et al.*, 2013) we did not perform such an injection control. Our bathing challenge was performed by adding the same amount of bacterial suspension (10<sup>6</sup> cells) to the beaker.

### 2.3 Experimental dissection and hemocyte analysis

The secondary *Vibrio* challenge experiment lasted for seven days in total. Mortality was checked daily. On day 1 and on day 7 post-challenge hemolymph samples were collected by withdrawing hemolymph from the posterior adductor muscle sinus of three randomly chosen mussels per treatment group using a 1-mL plastic syringe fitted with a 23-gauge hypodermic needle. Hemolymph samples were immediately transferred into 1.5 mL Eppendorf tubes held on ice to avoid hemocyte clumping. To determine *Vibrio* load in mussel hemolymph, five µl of undiluted hemolymph were spread out on petri dishes containing thiosulfate-citrate-bile salts-sucrose (TCBS)-agar and incubated over night at room temperature before CFU were counted. For hemocyte composition analysis, an aliquot of 100 µL hemolymph was fixed with an equal volume of 6 % formalin diluted in seawater. To identify mussel hemolymph cells, DNA was stained with SYBR-green I. 4 µL SYBR-I were added and incubated for 120 min in the dark at room temperature before flow cytometry analysis. Flow cytometry analysis was performed on a BD

Accuri™ C6 Flow Cytometer (BD, Biosciences). We gated cells according to their DNA content corresponding to the SYBR-I fluorescence. Cell populations were identified in a forward scatter (FSC) and side scatter (SSC) plot. Samples were run until a total of at least 500 events were measured in the gated hemocyte populations. All flow cytometric samples were checked for clear cell population differentiation and otherwise removed from the analysis. To measure activation of cellular immunity we measured phagocytotic activity. To do so 100  $\mu$ L of untreated hemolymph were mixed with an equal amount of 0.45  $\mu$ L fine-filtered sea water to avoid osmotic stress. Afterwards 10  $\mu$ L of 1 mM fluorescent beads (Sigma-Aldrich) were added and the tubes incubated for 120 min in the dark at room temperature to allow hemocytes to take up the beads. The reaction was stopped by adding 100  $\mu$ L of cold anti-aggregant solution (AASH, (Auffret & Oubella, 1994)). Cells that took up beads were identified by gating according to bead fluorescence and phagocytosis rate was calculated as the proportion of cells that phagocytosed beads from the whole cell population.

### 2.4. Statistical analysis

All statistical calculations were performed within [R], (R Development Core Team 2016). We fitted full models with the different infection treatments (*Vibrio* and *Mytilicola*), temperature treatments (ambient and elevated temperature) and time of analysis (day 1 and day 7) as independent predictors where possible, but removed higher order interaction terms to account for missing data resulting from mortality, (e.g. injected mussel treatment at day 7 and elevated temperatures). *Vibrio* load measured as colony forming units (CFU) was analyzed by negative binomially distributed Generalized Linear Models (GLMs) using likelihood ratios for significance testing. Mortalities were analyzed by non-parametric models based on Cox proportional hazards. Hemocyte composition was analyzed by permutational multivariate analysis of variance (Permanova, *adonis* function of the *vegan* package) using a euclidean distance matrix as response variable that was calculated from the proportion of each cell type. Phagocytotic activity of hemocytes was analyzed by binomial GLMs comparing the number of phagocytosing cells to the number of not phagocytosing cells as a function of our experimental treatments for each cell population, and we investigated the relationship between phagocytotic activity and *Vibrio* load by weighted regressions of means for each experimental group using the inverse standard error as a weighting variable.

## 3. Results

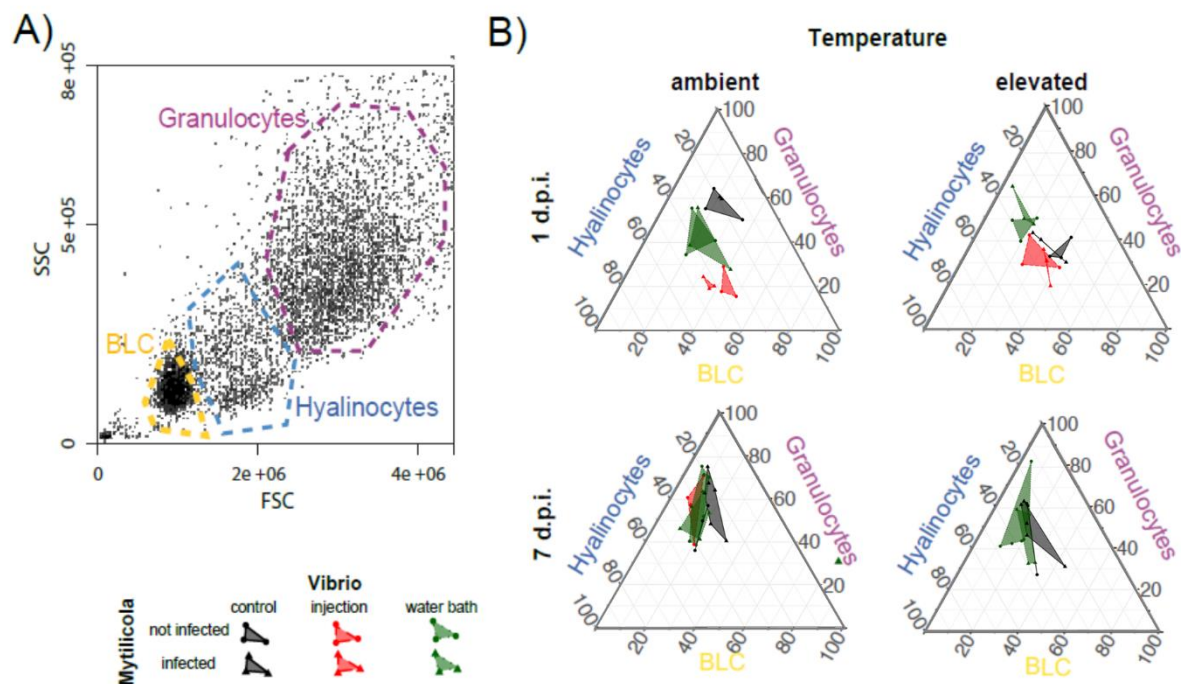
Parasites were dissected from mussels either when they died throughout the experiment or at the end of the experiment 7 days past *Vibrio* infection. We found parasites in 95 % of infected mussels with an average infection intensity of  $9.02 \pm 0.58$  parasites per mussel. In uninfected mussels, on the other hand, individual mussels had no or maximum one parasite. We only found two out of 60 mussels to be infected with more parasites (one with two parasites and one with 11 parasites probably representing a falsely



infected control mussel). This last individual was only used in the survival analysis. Similarly, we cannot exclude that exposed but uninfected individuals were never infected throughout the experiment and just expelled their parasites prior to dissection. Since the inclusion of exposed but uninfected and falsely exposed hosts is conservative towards our hypothesis, we decided to keep these individuals in the analysis. Removal of the falsely infected individual did not qualitatively change the results of the survival analysis (data not shown).

### 3.1. Hemocyte composition

Throughout the experiment, proportional composition of the three hemocyte sub-populations varied significantly with blast like cells decreasing and granulocytes increasing between day 1 and day 7 d.p.i., (Tab. Ch.III.1., Fig. Ch.III.1.). Next to temporal variation, the main experimental factor clearly separating hemocyte sub populations was *Vibrio* infection by significantly decreased the proportion of granulocytes and blast like cells (Fig. Ch.III.1., Tab. Ch.III.1.).



**Figure Ch.III.1.:** Flow cytometric scatter plot (A) of forward (FSC, measuring cell size) and sideward (SSC measuring cell complexity) scatter differentiating three hemocyte cell populations (blast like cells (BLC), Hyalinocytes and Granulocytes).

Proportional composition of the three hemocyte cell types (B) for day 1 and day 7 past infection (d.p.i.) for both experimental temperatures (left: ambient, right: elevated). Polygons include all samples from each experimental group with colors representing *Vibrio* infection treatments. Polygons with dots show mussels not infected with *Mytilicola* while triangles mark infected mussels.

### Chapter III

Grouping according to *Vibrio* infection treatment was strongest on day 1 but disappeared over time (significant time x *Vibrio* interaction in Tab. Ch.III.1., Fig. Ch.III.1.). Furthermore, especially at elevated temperatures in combination with *Vibrio* infection shifted hemocyte populations towards more granulocytes (significant temperature x *Vibrio* interaction in Table Ch.III.1., Fig. Ch.III.1.). Otherwise, temperature did not affect hemocyte composition and neither did parasite infection by itself nor in any combination with other treatments.

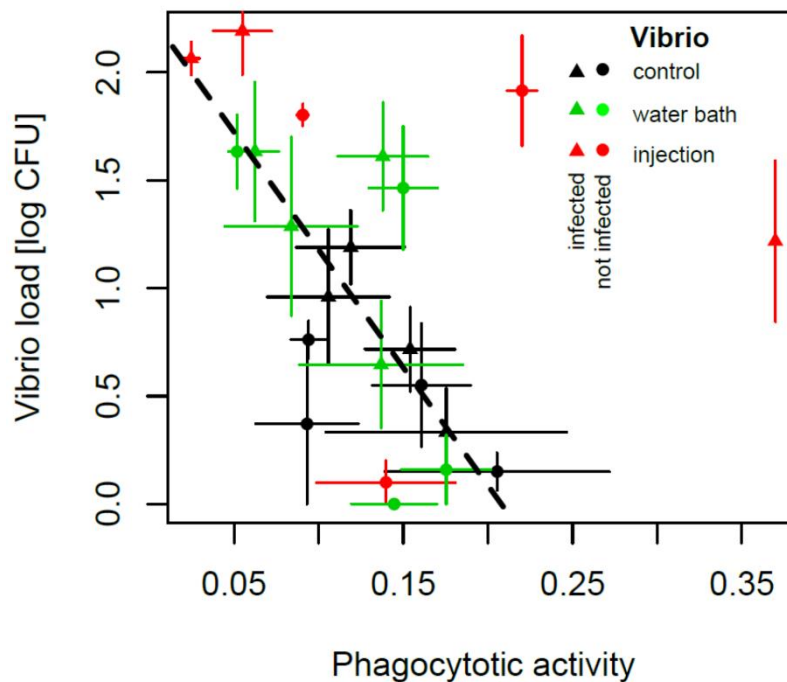
**Table Ch.III.1.:** Analyses of hemocyte composition, phagocytosis rate, infection intensity (i.e. total culturable *Vibrio* counts) and mussel survival.

Significant factors are highlighted in bold. \*\*\*  $p < 0.001$ ; \*\*  $0.001 < p < 0.01$ ; \*  $0.01 < p < 0.05$ ; (\*)  $0.05 < p < 0.1$ ; LRT = Likelihood ratio test; CFU = colony forming units, nf = term not fitted

Effect	Response				
	Hemocyte composition <i>PerMANOVA</i>	Phagocytosis <i>Binomial GLM</i>	<i>Vibrio</i> load [CFU] <i>negative binomial GLM</i>	Survival <i>Cox proportional hazard</i>	
	F	LRT $\chi^2$	LRT $\chi^2$	-	LRT $\chi^2$
Day (D)	<b>23.431</b> df=1 ***	<b>202.508</b> df=1 ***	<b>12.920</b> df=1 ***	2*Loglikelihood NULL = -232.95	
<i>Vibrio</i> infection (V)	<b>6.327</b> df=2 **	<b>148.102</b> df=2 ***	<b>9.933</b> df=2 **	<b>-205.58</b>	<b>54.746</b> df=2 ***
<i>Mytilicola</i> infection (M)	0.168 df=1	<b>6.599</b> df=1 *	0.015 df=1	<b>-201.30</b>	<b>8.547</b> df=1 **
Temperature (T)	2.311 df=1	<b>5.828</b> df=1 *	0.673 df=1	<b>-198.34</b>	<b>5.932</b> df=1 *
D x V	<b>4.154</b> df=2 **	<b>195.583</b> df=2 ***	<b>13.993</b> df=2 ***	nf	
D x M	0.440 df=1	<b>6.557</b> df=1 *	<b>23.283</b> df=1 ***	nf	
D x T	0.618 df=1	<b>16.815</b> df=1 ***	<b>4.665</b> df=1 *	nf	
V x M	0.550 df=2	<b>146.785</b> df=2 ***	1.529 df=2	-197.50	0.630 df=2
V x T	<b>2.692</b> df=2 *	<b>35.352</b> df=2 ***	0.300 df=2	-195.92	3.171 df=2
M x T	0.529 df=1	<b>93.133</b> df=1 ***	0.007 df=1	-195.77	0.298 df=1
V x M x T	nf	nf	nf	<b>-193.37</b>	<b>4.797</b> df=2 (.)

### 3.2. Phagocytosis and *Vibrio* load

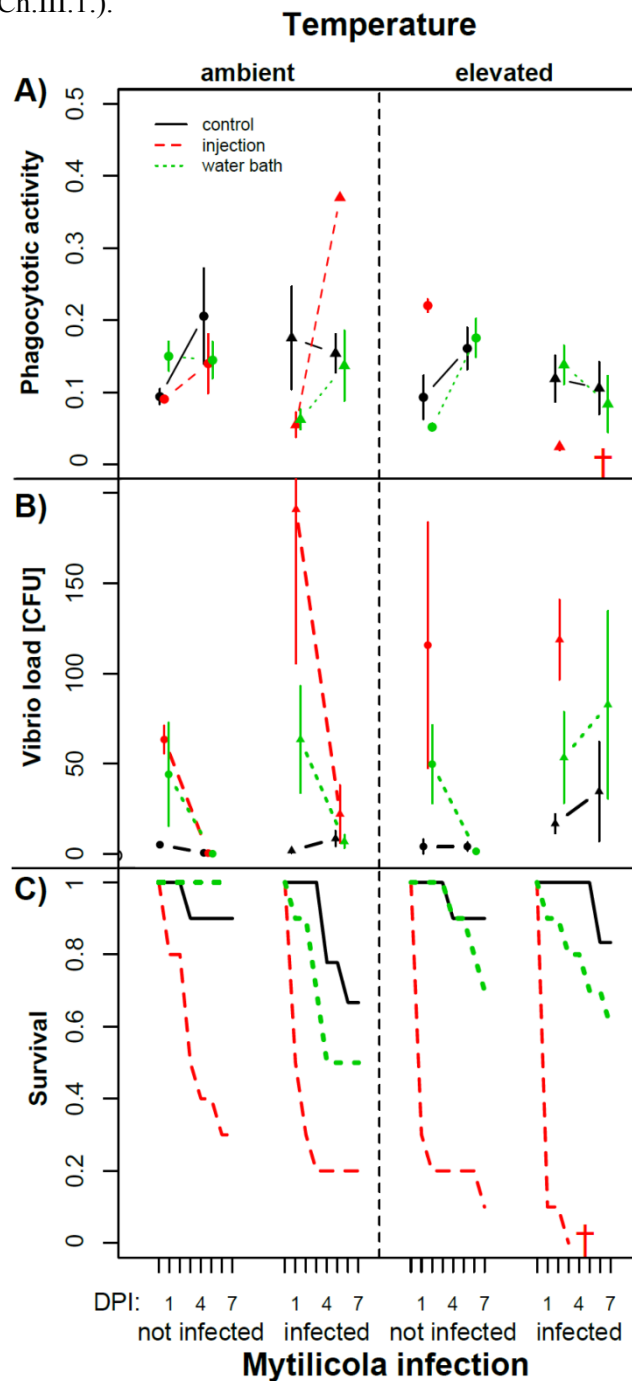
Granulocytes did not only show the strongest shift in hemocyte composition, but also contributed most to phagocytosis with an average activity of 13.03% compared to 3.58% and 1.12% for Hyalinocytes and Blast like cells, respectively. We therefore concentrate our analysis of phagocytotic activity on granulocytes as the main cell population interacting with *Vibrio* in the hemolymph. Granulocyte phagocytosis was overall a good predictor for *Vibrio* load indicating phagocytotic activity controls *Vibrio* load in the hemolymph (Fig. Ch.III.2.,  $F_{1,21} = 4.52$ ,  $p = 0.047$ ).



**Figure Ch.III.2.:** Relationship between mean phagocytotic activity (proportion of phagocytotically active cells  $\pm$  1 SE) and mean *Vibrio* load per experimental group ( $\pm$  1 SE). Colors code for the different *Vibrio* infection treatments while triangles represent groups with and circles groups without *Mytilicola* infection.

Two groups deviated quite strongly from the equilibrium ratio (*Vibrio* injection of *Mytilicola* infected mussels on day 7 at ambient temperature and *Vibrio* injected mussels without *Mytilicola* infection on day 1 at elevated temperature). The fact that both groups belonged to the *Vibrio* injection treatment might suggest that injection delivered more *Vibrio* directly into the hemolymph for which it took longer to clear and reach the equilibrium ratio between load and phagocytotic activity. Removal of the two obvious outliers from the injection treatment (Fig. Ch.III.2.) improved the fit substantially ( $F_{1,19} = 24.8$ ,  $p < 0.001$ ). When looking at the dynamic relationship between *Vibrio* load and phagocytotic activity over time (Fig. Ch.III.3., A/B) it becomes obvious that the high phagocytotic activity of mussels infected with *Mytilicola* at ambient temperatures on day 7 is a direct response to high *Vibrio* loads on day 1 in that group. Similarly, high *Vibrio* load in uninfected mussels on day 1 at elevated temperatures elicited a strong immune response that was nevertheless not efficient enough to control *Vibrio* populations,

(Fig. Ch.III.3., A/B). While both of our infection treatments increased *Vibrio* load on day 1 after infection, parasite infection further increased *Vibrio* load on day 1 especially at ambient temperatures, (Fig. Ch.III.3., B; Tab. Ch.III.1.).



**Figure Ch.III.3.:** A) Temporal dynamics of phagocytotic activity expressed as the proportion of bead phagocytizing granulocytes on day 1 and day 7 after *Vibrio* infection at ambient temperatures (left panel) and elevated temperatures (right panel).  
 B) *Vibrio* load in the hemolymph expressed as colony forming units [CFU] in 5  $\mu$ L of hemolymph.  
 C) Daily survivorship of mussel in the different experimental groups. Each panel compares mussels infected with *Mytilicola intestinalis* to uninfected mussels with colors coding for the different *Vibrio* infection treatments.

At ambient temperatures, the activation of phagocytosis resulting from infection was however capable to control *Vibrio* load by day 7 and reduce it to control levels (Fig. Ch.III.3., A/B). Elevated temperature also led to an increased *Vibrio* load over time (Tab. Ch.III.1.), probably reflecting the higher growth rates of *Vibrio* in this environment. Without *Mytilicola* infection mussels were however still able to mount an immune response and control *Vibrio* load by day 7 (Fig. Ch.III.3., B). The combination of *Mytilicola* infection and elevated temperatures, on the other hand, did not lead to increased phagocytic activity and consequently resulted in increased *Vibrio* loads and failure to clear *Vibrio* from the hemolymph by day 7 even in the control treatment (Fig. Ch.III.3., A/B).

### 3.3. Mortality

Out of 120 mussels 52 died during the one week challenge (Fig. Ch.III.3., C). Drawing hemolymph had no significant effect on mussel mortality (cox proportional hazard model,  $\chi^2 = 1.414$   $p = 0.234$ ). The main factor decreasing survival was *Vibrio* infection especially by injection, (Fig. Ch.III.3., Tab. Ch.III.1.). Additionally, *Mytilicola* infection amplified mortality probability, but did not significantly interact with the secondary *Vibrio* infection suggesting additive rather than synergistic effects in general, (Tab. Ch.III.1.). While temperature had a negative effect on survival in general, we only observed a marginally significant three-way interaction with the infection treatments reflecting the strong increase in mortality in the *Vibrio* bath treatment when mussels were infected by *Mytilicola* at ambient temperatures (Tab. Ch.III.1., Fig. Ch.III.3.).

#### 4. Discussion

The uncontrolled invasion of alien species and the concomitant loss of biodiversity harbors a potential risk of increasing disease transmission in recipient ecosystems (Keesing *et al.*, 2010). Invasive parasites directly and indirectly contribute to this aspect of invasions (Dunn, 2009), since parasite spillover can directly lead to the emergence of diseases in native biota (Goedknecht *et al.*, 2016) or indirectly favor secondary infections (Iglesias *et al.*, 2015). The mass mortalities of blue mussels *M. edulis* shortly after the spill over of the parasite *M. intestinalis* (Blateau *et al.*, 1992; Korrynga, 1951; Meyer & Mann, 1950) represent a good example for strong effects on the newly acquired host species resulting from a parasite invasion (Goedknecht *et al.*, 2016). Evidence for the direct involvement of *M. intestinalis* in these mortalities remained however circumstantial. In general, *M. intestinalis* seems not to have serious direct pathological impacts on its mussel host (Bower *et al.*, 1994) suggesting that potential indirect effects with increased severity of secondary infections may also have played a role here. Accordingly, our experimental infections showed that the primary infection of an invasive parasite can amplify the trait mediated negative indirect effects of a parasite invasion by modulating the cellular immune response leading to increased severity of secondary infections. Since these trait mediated indirect effects also fed back on host survival, other density dependent indirect effects affecting other species in the community can follow (Hatcher *et al.*, 2012).

The secondary *Vibrio* infection was the main cause for mortality especially when *Vibrio* was injected at higher temperatures (Fig. Ch.III.3., C). While parasite infection significantly decreased survival in all treatments, we could not observe synergistic effects leading to disproportional mortalities in co-infections. Only in the water bath *Vibrio* infection at ambient temperatures, survival was considerably decreased when mussels were previously infected by *Mytilicola* (see marginally significant three-way interaction of the survival analysis, Tab. Ch.III.1.). This might indicate that the easier entry path generated by the parasite infection could have an effect when *Vibrio* cell division rate is low and *Vibrio* cells are mainly taken up from the environment. By using direct injection as well as a water bath for *Vibrio* infection, we could differentiate between different barriers of defense (Schmid-Hempel & Ebert, 2003). The hooklike structures, with which the parasite is clinging to the gut cause considerable damage of the mussels' intestinal gut wall (Hockley, 1951; Moore *et al.*, 1978). With higher temperatures or other unfavorable conditions (e.g. anoxia), parasites get more active (Grainger, 1951; Korrynga, 1968). Such increased parasite movement will cause more gut wounds since the parasite needs to repeatedly attach to the gut wall. Although repair is generally rapid (Moore *et al.*, 1978), the permanent penetration of the gut wall could provide an easy entry pathway for *Vibrio* or other opportunistic pathogens. Easier pathogen entry should first of all correlate to increased *Vibrio* load in the hemolymph of mussels infected with *Mytilicola* in water bath infections. The comparison with uninfected mussels however showed no elevated *Vibrio* load in infected mussels (Fig. Ch.III.3., B), indicating that ease of entry was not the main reason for higher *Vibrio* loads and increased mortalities. Parasite infection nevertheless increased *Vibrio*

load in injection treatments indicating that downstream processes after initial infection played a more predominant role.

One such process could be that parasite infection alters the immune response profile of infected mussels. While mussels can mount a cellular immune response by engulfing or encapsulating parasites and pathogens in the hemolymph (Galimany *et al.*, 2008), *M. intestinalis* occurs in the gut and is too big to be controlled by hemocytes directly. Consequently, the production of reactive oxygen species (ROS) is the predominant immune effector in a *Mytilicola* infection (Feis *et al.* submitted). Although hemocytes are not directly involved in a *Mytilicola* immune response, the efficiency of a cellular immune response to the secondary bacterial infection was nevertheless affected. On day 7 all the non-parasitized mussels were able to clear most *Vibrio* and reach levels comparable to the control treatment and the decreased phagocytosis rates lead to a slower clearance or even failure to clear of *Vibrio* from the hemolymph only in *Mytilicola* infected mussels (Fig. Ch.III.3.). The control of *Vibrio* concentration in the hemolymph by phagocytosis is known to be the principal immune response to bacterial infections in bivalves (Labreuche *et al.*, 2006; Pruzzo *et al.*, 2005; Wendling & Wegner, 2015) and the correlation between *Vibrio* load and phagocytotic activity observed here (Fig. Ch.III.2.) underlines that the secondary infection is controlled by the cellular immune response. Functionally higher bacterial load thus seems to be linked to impaired phagocytosis of hemocytes. Such immune depression is commonly observed in coinfections (Cornet *et al.*, 2009; Graham, 2008) but the physiological basis can vary with host-parasite pairs (Cornet *et al.*, 2010). One possibility is that fewer resources are available for the cellular immune response when challenged by a primary infection that binds resources for a ROS immune response against the parasite. Since resource availability in bivalves is tightly connected to nutrition (Chen *et al.*, 2005; Delaporte *et al.*, 2003), not only the immune response against the *Mytilicola* infection limits resource availability, but the parasite might also directly impair the digestive function and nutrient uptake by damaging gut tissue or altering microbial gut flora. The constant presence of *M. intestinalis* within the intestine and its impact on surrounding tissues may therefore generally disturb the host in its immunological vigor.

Decreased hemolymph clearance can also be the result of less phagocytotically active cells in parasitized mussels. Since we could not detect any significant effect of *Mytilicola* infection on the proportional composition of hemocytes, a direct effect of hemocyte population shifts on phagocytosis seems unlikely. Nonetheless damaging the epithelial gut cells will cause a constant hemocyte infiltration into the connective tissues (Figueras *et al.*, 1991; Villalba *et al.*, 1997), which may affect the adjustment and availability of hemocytes for immune defense processes in the hemolymph. Hyalinocytes are the main cell population involved in wound repair and are consequently less phagocytotically active (Aladaileh *et al.*, 2007). Since the immediate bivalve immune defense relies on fast tissue infiltration (Le Foll *et al.*, 2010), which we also observed for *Vibrio* infection treatments, the permanent emission of

hyalinocytes into the sites of *Mytilicola* infection probably increases the energetic demands for maintaining hemocyte homeostasis (Renwantz *et al.*, 2013) in the hemolymph and might additionally limit the number of phagocytotically active cells in the hemolymph.

The immune activity of bivalves and particularly the phagocytotic activity is usually negatively correlated with stressors (Coles *et al.*, 1995; Fournier *et al.*, 2001). The failure to control *Vibrio* load in the hemolymph in infected mussels at elevated temperatures correlated to a decrease in phagocytotic activity which might indicate that the amount of dead or inactive hemocytes is also increased in stressful conditions. That an increase of *Vibrio* load at elevated temperatures was also observed in the control treatment where no additional *Vibrio* were added underlines the overall disability in mounting an effective immune response. *Vibrio* spp. are generally enriched in the hemolymph compared to seawater, (Lokmer *et al.*, 2016) and in the absence of external *Vibrio* such accumulation will be amplified in the hemolymph of immunologically impaired mussels, especially at elevated temperatures where the dynamics of *Vibrio* accumulation and within host growth will be accelerated.

In conclusion, we could show that *M. intestinalis*, influences secondary infection rates by modulating the cellular immune response and is therefore indirectly affecting the pathogenesis of *Vibrio* spp. infections. Although the indirect effect of immunosuppression can be expected for coinfections, (Graham, 2008; Woolhouse *et al.*, 2015) only few empirical studies investigated this effect in the context of invasive species (Cornet *et al.*, 2009) and could link trait mediated to potential density mediated effects that we can postulate as a result of our study. Consequences of the direct effects of *M. intestinalis* on its host like tissue damage and hemocyte infiltration into surrounding tissues could thereby limit the efficacy of the anti-bacterial immune response of the mussel host. When *M. edulis* is additionally affected by secondary infections or under adverse environmental conditions like high temperatures, parasite infection indirectly increase the mortality rate of its host and elicit further density dependent indirect effects (Hatcher *et al.*, 2012). Due to its particularly immunological vigor (Wootton *et al.*, 2003), most of *M. edulis* mortalities were attributed to synergetic effects of physiological attenuation resulting from unusual and adverse biotic or abiotic conditions, (Freeman & Dickie, 1979; Incze *et al.*, 1980; Worrall & Widdows, 1984). Consequently, previous mortalities were attributed to adverse temperatures in combination with reproductive stress as well as genomic and cytogenetic abnormalities (Béchemin *et al.*, 2015; Benabdelmouna & Ledu, 2016; Myrand *et al.*, 2000).

Although, we found increased mortality of mussels infected with *Mytilicola* this effect could still be indirect and result from a decreased bacterial clearing rate due to lower phagocytotic activity of infected mussels. This may suggest that the reported mortality events of *Mytilicola*-infected blue mussels were not solely caused by this parasite but that parasite infection could merely have favored an outbreak of disease. However, there are many examples of mussel populations in the North Sea, that are infested



with *M. intestinalis* in high intensities without showing any mass mortalities, (Elsner *et al.*, 2011; Pogoda *et al.*, 2012). While our results suggest that the permanent direct effects like the energetically costly hemocyte infiltration might weaken the immune response of *M. edulis*, further stressors might be needed to invoke high mortality rates. Nevertheless, our results clearly demonstrate that parasite invasions can have profound trait-mediated indirect effects by weakening the immune response towards secondary infections. With the projected changes in temperature increase or ocean acidification in the marine environment (Mackenzie *et al.*, 2014b) such trait- and density dependent indirect effects of parasite invasions can be amplified and need to be considered to assess the overall effect of species invasions.





### III. Conclusions and Perspectives

Direct and indirect effects of invasive parasite infections can render the new host more susceptible to further impacts like climate change or secondary infections (Hatcher *et al.*, 2012; Studer *et al.*, 2010). Consequently, invasive parasites threaten to modify ecological interactions within communities and to increase the risk of disease emergence (Peeler *et al.*, 2011). Driven by the need to understand the ecological consequences of parasite mediated invasion processes, the present study was designed to investigate the **direct and indirect effects** of an invasive copepod parasite *Mytilicola intestinalis* on a native bivalve *Mytilus edulis* making explicitly use of a direct lifecycle parasite / native host system.

In the German Wadden Sea, blue mussels *M. edulis* have experienced an invasion of the parasite *M. intestinalis*. The parasite directly limits the hosts' resources for growth (Chapter I) and for the immune response against secondary infections (Chapter III). The resulting indirect effects can be more serious, as they can act cumulatively with further stressors, e.g. raised temperatures (Marcogliese, 2008) and can increase the mortality risk for the host (Chapter II and III).

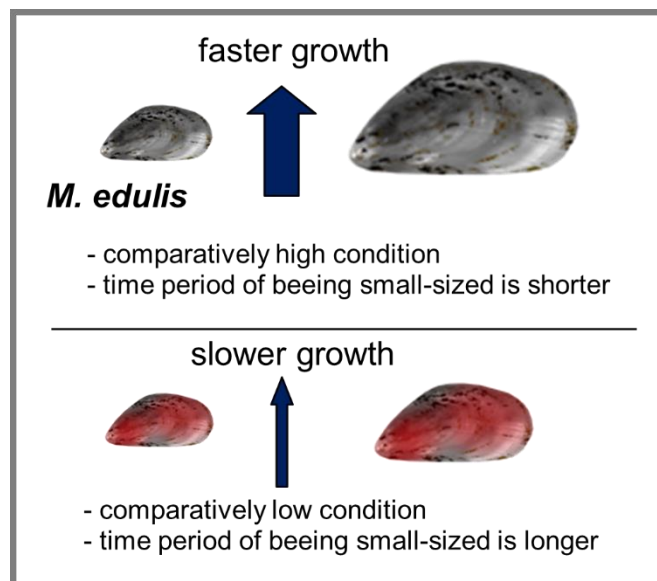
Anthropogenic climate change is expected to increase the occurrence of such biotic and abiotic stressors that can intensify the negative effects of host parasite interactions. Among others, these include ocean warming and acidification (Harvey & Moore, 2017) as well as the new species invasion. For example, *Mytilicola orientalis*, a parasite related to *M. intestinalis* and that is on the list of the 100 'worst invasive' species in the Mediterranean Sea, is likely to spillover from invasive oysters to native blue mussels, (Goedknecht *et al.*, 2017; Stock, 1993; Streftaris & Zenetos, 2006). Such events could have long-term consequences not only for the individual host but for the mussel population and the whole ecosystem as well. Deep understanding of the effects of invasive parasites requires application of various experimental approaches across multiple scales (Papacostas *et al.*, 2017) to assess future consequences.

In this study, I applied multi-disciplinary methods to examine the direct (Chapter I) and the indirect effects (Chapter II and III) of *M. intestinalis* on *M. edulis*. I investigated both short and long-term consequences of controlled parasite infections on mussels, by combining field and laboratory experiments. Simultaneously, I observed the parasite incidence of a heavily infected wild mussel population on the Island Sylt over the course of one year.

I found that the parasite primarily feeds on host tissue and not on ingested food accumulated within the host's gut, as indicated by its higher trophic level compared to the host (Chapter I). As a consequence, the metabolomic profile between parasitized and non-parasitized mussels was clearly separated for the foot and adductor muscle but not for the gills and mantle. Whereas I found no significant difference in the shell strength between parasitized and non-parasitized mussels, I could demonstrate a lower growth rate for parasitized mussels (Chapter I). Furthermore, I found indirect effects of *M. intestinalis* affecting the predator-prey relationship between mussels and shore crabs as well as the immune response of the host against secondary *Vibrio* infections (Chapter III).

### 1. Direct non-lethal effects of *M. intestinalis* on *M. edulis*

An infection with *M. intestinalis* does not lead to a metabolic suppression for all tissues but it does disturb the general metabolic homeostasis and decreases condition of parasitized mussels (Chapter I). Thus, parasitized mussels maintain essential life-sustaining activities like the production of shell material by mantle tissue (no differences in shell strength and no clear separation in the metabolomic profile of the mantle tissue between parasitized and non-parasitized mussels, Chapter I). Consequently, the direct effects of *M. intestinalis* do not result in mortality for the host.



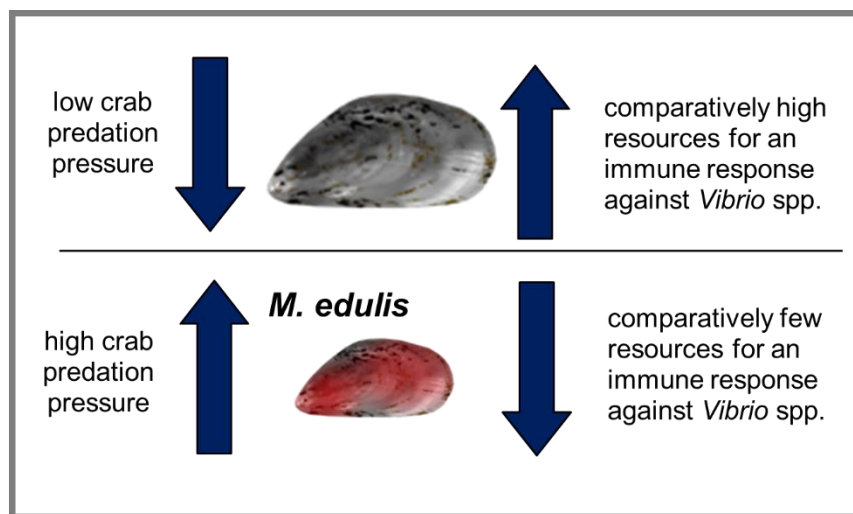
**Figure III.1.:** Life history traits (growth and condition) of non-parasitized (grey) and parasitized (red) mussels.

Parasitized mussel individuals show a lower condition that is expressed in a decreased growth rate, (Chapter I).

However, the direct effects of *Mytilicola* infection on condition and growth can have ecological consequences for mussels. The negative effects on mussel condition caused by *M. intestinalis* can adversely affect other important functions that are not only important for the host itself but for associated species as well, such as altered filtration rates (Meyer & Mann, 1950; Sousa *et al.*, 2009; Stier *et al.*, 2015). Additionally, a weaker adductor muscle and reduced growth can raise the predation pressure by sea stars or crabs that prefer smaller size classes as prey (Hancock, 1965; Juanes, 1992). Parasitized mussels also stay longer in a smaller size class due to a reduced growth rate, further increasing the risks mentioned above for parasitized mussels (Chapter I). Besides, small mussels mature later, reproduce less and contribute less to the gene pool (Suchanek, 1981). The negative direct effects of *M. intestinalis* are manifested in reduced condition and growth resulting in negative indirect effects, namely higher susceptibility of parasitized mussels to adverse environmental impacts like food deprivation or predation compared to the non-parasitized mussels of the same age.

## 2. Will indirect effects lead to an increased mortality of mussels?

I found that the predation by '*Carcinus maenas*' on mussels was affected in relation to different parasite stages (Chapter II), either increasing or decreasing the predation pressure on parasitized mussels. Besides, my results show that *M. intestinalis* can indirectly affect the hosts' cellular immune response. Specifically, the hemocytes of parasitized mussels exhibited impaired phagocytotic functions which was in turn correlated with a higher *Vibrio* load (Chapter III). This effect was the strongest in parasitized mussels that were additionally infected with *Vibrio* and exposed to high temperatures. Thereby, I could demonstrate that indirect effects of an infection with *M. intestinalis* can lead to an increased mortality of *M. edulis* (Chapter II and III).



**Figure III.2.:** Indirect effects of *Mytilicola* infection on the interaction between *M. edulis* and *C. maenas* as well as *Vibrio* spp. for non-parasitized (grey) and parasitized (red) mussels. Parasitized mussel individuals (infected with adult parasite stages) show a higher susceptibility to crab predation, (Chapter II). Further, a parasite infection can indirectly increase the severity of secondary *Vibrio* infections within mussels (Chapter II).

The risk of increased mortality for parasitized mussels either due to increased predation pressure by crabs or due to an increased susceptibility against secondary *Vibrio* infections may have further ecological consequences for parasitized mussels. Even if *M. intestinalis* (when still sexually immature) decreases crab predation on mussels (Chapter II), this would indirectly increase the prevalence of the parasite within a mussel population and intensify the direct negative effects of *M. intestinalis*, (Chapter I). The decreased condition and decreased phagocytotic activity (Chapter I and III) may push parasitized mussels past a point of recovery, when secondary infected, much faster than non-parasitized mussels (Renault *et al.*, 2005). As a conclusion, although *M. intestinalis* by itself is not likely to cause host mortality, it is very likely to indirectly contribute to increased mortality rates, in combination with further stressors.

### **Ecological relevance of *Mytilicola* infection and disease risk for the host**

*Mytilus edulis* is an important ecosystem engineer whose abundant shells provide extensive beds as an existential basis for a high diversity of species (Buschbaum *et al.*, 2009; Gutierrez *et al.*, 2003). Shells not only provide space of settlement for associated species, mussels can also use rapid growth rates to maintain spatial dominance (Petraitis, 1995). However, growth rates depend on a complex pattern of environmental factors e.g. temperature, food availability, seawater salinity and tidal height (Bergström & Lindegarth, 2016; Buschbaum & Saier, 2001) and can vary dependently. The effect of parasite infection on mussel growth might be lower than that of other factors mentioned above. Nevertheless, *M. intestinalis* can function as an overlaid stressor and the observed direct effects reveal a hidden determinant of mussel growth induced by parasite infection (Chapter I).

Besides providing habitat for many species via production of shells *M. edulis* has a number of further ecological functions that determine community composition. By accelerating secondary production rates, mussels show high biomass amounts and not only accelerate nutrients for benthic species (Asmus, 1987; Kautsky & Evans, 1987) but also constitute an essential food source for crabs and birds (Nehls *et al.*, 1997). If mussel populations would decline then a number of associated species in the coastal ecosystem would likewise suffer.

Generally, *M. edulis* is relative resistant to bacterial pathogens related to its robust innate immunity, (Watermann *et al.*, 2008; Wootton *et al.*, 2003). Contrarily to oysters (for example *Magallana gigas*), very few mass mortalities of blue mussels occurred in the past (Polsenaere *et al.*, 2017). Nevertheless, on the immunological level, bivalve health is at a risk because elevated temperatures can not only facilitate the proliferation of pathogens (Harvell *et al.*, 2002), but haemocyte functionality of mussels can also be strongly affected by higher temperatures, ocean acidification and altered salinity (Mackenzie *et al.*, 2014a; Matozzo *et al.*, 2012). Although the relative effect on phagocytotic activity of *M. intestinalis* infection might not be a root factor causing mortality, it can nevertheless additively compromise mussel immunity and aggravate diseases (Chapter III). High bivalve die offs are often driven by several adverse factors that occur simultaneously (Renault *et al.*, 2005). Thus, any factor that impairs phagocytotic ability of mussel hemocytes could contribute to insufficient clearance of bacteria and mussel mortality. Consequently, the *Mytilicola*-caused reduction of phagocytotic function should be considered when evaluating possible causes of ultimate mortality for mussels. Furthermore, the revealed **direct and indirect effects** of *M. intestinalis* on *M. edulis* (Chapter I-III) may be transferable to other bivalve/copepod systems with the potential of having equal consequences for the parasitized hosts.

### Limitations and perspectives

Within the following section, I would like to address some limitations of my thesis and make recommendations for future research.

Concerning the measurement of condition, I met several difficulties. Whereas I found significant differences in condition between experimentally infected and control mussels, I could not demonstrate this relationship in wild mussels (Chapter I). The static ratio between dry weight and cubed shell length may not be sensitive enough for measuring condition within wild mussels, because it only reflects a snapshot of the measured parameters regardless of acute biotic and abiotic factors that might have affected the mussels differently. The results can thus show higher variations between species than variations due to body condition. Dynamic indices based on production estimates over a certain time period (Lucas & Beninger, 1985) are less prone to the difficulties mentioned above. Thus, growth might be a better proxy for mussel condition reflecting physiological activity over time. Other parameters for example glycogen content / lipids / ion concentrations, may additionally provide insights into the mechanisms behind the loss of body condition and energy demand within the *M. intestinalis* / *M. edulis* system in the future studies.

In my thesis, I show that *M. intestinalis* changes the predator-prey interaction between the mussels and shore crabs (Chapter II). Whether *M. intestinalis* actively manipulate or indirectly weaken the host cannot be answered at present. So far, I found no effect of *M. intestinalis* infection on the shell strength (Chapter I) and no difference in handling time between parasitized and healthy mussels by predatory crabs (Chapter II). This may indicate that active manipulation of the host by the parasite might have taken place. Evaluating the pure handling time (without intermission times) may yield more precise results in this respect. Future research analyzing the relative contribution of direct behavior manipulation and indirect side effects could reveal possible underlying mechanisms.

Predator-prey relationships are very important in structuring dynamics of communities (Hatcher *et al.*, 2014). I assume the effects shown in this study (Chapter II) to be highest within mussel beds mixed of uninfected and parasitized mussels respectively when crabs have a choice. Generally, the crabs' prey preference is dependent on many factors like mussel size or the presence of associated organisms like eubionts (Enderlein *et al.*, 2003). Furthermore, the impact of parasitism on the host can also be affected by altered environmental conditions, like higher temperatures (Larsen & Mouritsen, 2009). Future predation experiments that use mussels which combine those factors (parasitism and size differences) with the experiments conducted under different temperature conditions could reveal insights into the magnitude of *Mytilicola* infection in relation to other factors influencing crabs' prey choice and the predator-prey interaction between *C. maenas* and *M. edulis*. The indirect effects of *M. intestinalis* on crabs' prey choice should be included into future research when assessing the prey preference of crabs within mussels where this parasite is involved.

The secondary infection of mussels with *Vibrio* revealed negative indirect effects of *M. intestinalis* on the mussels' immune response (Chapter II), offering further perspectives to investigate

the effect of parasite infection on mussel diseases. The presence of *M. intestinalis* in the gut for example is likely to affect the structure of mussel microbiota. Changes in microbiota diversity and composition may affect the resistance to colonization by external pathogens and facilitate secondary infections. The link between the parasite, microbiota and mussel fitness (Lokmer *et al.*, 2016) thus represents an interesting question for future studies.

One task for marine science is to estimate the direct and indirect effects of invasions for native species with respect to future environmental challenges (e.g. ocean warming). *M. intestinalis* and its direct and indirect effects, as well as the mussel hosts and the interacting species can be affected by a change of the biotic and abiotic factors. So far, the mussel populations in the Wadden Sea have been spared from mass mortalities despite the high prevalence of the parasite since more than 30 years. Nevertheless, I predict that the parasitized mussels will be at a clear disadvantage compared to non-parasitized mussels, based on my findings concerning energy resources for growth and immune response. The negative direct and indirect effects of *M. intestinalis* for the host may become more important according to the challenges of future climate change.



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## **Affidavit**

Herewith I affirm that the work in hand entitled:

**‘Direct and indirect effects of invasive parasites on native blue mussels’**

is apart from my supervisor’s guidance and the cooperation with my co-authors the result of my own work. Furthermore, I confirm that this thesis has not yet been submitted (neither partly nor completely), as part of another examination process.

This thesis has been prepared in compliance with the rules of Good Scientific Practice of the German Research Foundation.

Hiermit bestätige ich, dass die vorliegende Abhandlung mit dem Titel:

**“Direct and indirect effects of invasive parasites on native blue mussels”**

abgesehen von der Beratung durch meinen Betreuer nach Inhalt und Form die eigene Arbeit ist. Ebenso bestätige ich, dass die Arbeit weder ganz noch zum Teil schon einer anderen Stelle im Rahmen eines Prüfungsverfahrens vorgelegen hat, veröffentlicht worden ist, oder zur Veröffentlichung eingereicht wurde.

Die vorliegende Arbeit ist unter Einhaltung der Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft entstanden.

Felicitas Demann