An underwater photograph showing a dense cluster of blue mussels on a rocky substrate. The mussels are dark blue with some lighter, yellowish-green patches. The water is slightly turbid, and the background is a soft, out-of-focus blue-green. The text is centered over the image.

**Host–parasite co-evolution and co-infection  
of invasive parasitic *Mytilicola* copepods  
in blue mussel hosts**

Marieke Eveline Feis

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Host–parasite co-evolution and co-infection  
of invasive parasitic *Mytilicola* copepods  
in blue mussel hosts

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Dedicated to my late father



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General introduction

**Marieke E. Feis**



## **On host–parasite co-evolution and why to study it**

Parasites are ubiquitous, which is highlighted by the fact that more than half of all organisms are parasitic (de Meeûs & Renaud 2002; Dobson *et al.* 2008; Morand 2015). They contribute substantially to the biomass and the energy flow in ecosystems (Kuris *et al.* 2008; Lagrue & Poulin 2016; Preston *et al.* 2013). Parasites impose natural selection through the fitness cost they cause in their host. The first step is infection of the host; next, extraction of the host resources; and finally, propagation and transmission to the next host. In return, hosts impose selection on parasites by keeping them out, i.e., resisting infection, or if that failed, by keeping the parasite's damage to a minimum, and by interfering with the parasite's reproduction, i.e., tolerating the infection to keep the fitness cost of parasitism to a minimum. These antagonistic interactions between host and parasites lead to an arms race between the parasite's infectivity and virulence, and the host's resistance and tolerance. Because parasites often have short generation times and large population sizes, they can evolve rapidly. The strong selection imposed on the host in turn elicits a rapid evolutionary adaptive response of the host toward the parasite. The result is the process of host–parasite co-evolution, ergo, a dynamic process that is based on the rapid reciprocal adaptation of both antagonists in response to the natural selection imposed by each of the antagonists.

Host–parasite co-evolution can thus be studied to understand rapid adaptation and evolution, and parasites offer a good manipulative system with choices of host–parasite combinations across the tree of life (de Meeûs & Renaud 2002). There is an interest in how parasitism and host–parasite co-evolution have shaped and are shaping biodiversity, and influence ecological specialisation and speciation processes (de Meeûs *et al.* 1998). Further, sexual reproduction has been suggested to have evolved as an adaptation to prevent parasite infections (Hamilton *et al.* 1990) and there is empirical support (Jokela *et al.* 2009; King *et al.* 2009; Lively 1987), as well as experimental support for this (Morran *et al.* 2011). Also disgust has evolved as an adaptive system for disease avoidance behaviour (Curtis *et al.* 2011). Moreover, there is an interest in predicting parasitism and disease spread for health and food stock. For instance, the biological invasion of the parasitic copepod *Mytilicola intestinalis* was associated with mass mortalities of farmed and wild mussels in the Netherlands (Stock 1950), leading to severe economic costs in the early 1950's, with a disastrously

low sale of only 5 million kg down from 40 to 50 million kg mussels (Drinkwaard 1993). Mass mortalities of mussels were also observed in France at the same time as high prevalence and intensities of *M. intestinalis* were recorded, with a decrease in mussel production from 10 000 tonnes in 1981 to 4000 tonnes in 1984 (Blateau *et al.* 1992).

## **On biological invasions and why to study them**

Invasive species are species that do not naturally occur in a geographic region, and can be described as alien, exotic, immigrants, colonizers, introduced, imported, translocated (see Colautti & MacIsaac 2004). Essentially, they are non-native and spread further into their novel range of their own accord. Valéry *et al.* (2008) define biological invasions as follows:

“ *A biological invasion consists of a species’ acquiring a competitive advantage following the disappearance of natural obstacles to its proliferation, which allows it to spread rapidly and to conquer novel areas within recipient ecosystems in which it becomes a dominant population.* ”

Biological invasions can have great impacts on human health, the economy, and are, in general, considered a global threat to biodiversity, natural ecosystems and agriculture (Early *et al.* 2016; Millennium Ecosystem Assessment 2005; Paini *et al.* 2016; Pimentel *et al.* 2001). Ecological impacts of invasive species include, but are not limited to, extinction, habitat alteration such as changes in energy and nutrient fluxes, and spread of parasites and diseases (e.g., Ehrenfeld 2010; Gallardo *et al.* 2015; Goedknecht *et al.* 2016; Reaser *et al.* 2007). While not all invasions are characterised by negative impacts, following the precautionary principle, new invasions are unwanted and should be prevented (Reise *et al.* 2006). The rate of biological invasions is ever increasing, as the world has gotten more and more connected through flights, shipping and other transportation (Cohen & Carlton 1998; Jackson & Grey 2012; Levine & D'Antonio 2003). For instance, the parasitic copepod *Mytilicola orientalis* co-invaded with translocated Pacific oysters in North America and Europe (His 1977b; Odlaug 1946) and was put on the list of the worst Mediterranean invaders (Zenetos *et al.* 2005).

Invasive species are studied through monitoring, early warning, rapid response and subsequent eradication programs to prevent establishment and spread of new invasive alien species, so that further loss of global biodiversity can be avoided. They are studied for their potential far-reaching impacts in their novel ranges, which can shed light on ecological and evolutionary interactions. Indeed, they can be used as a model to study ecological and evolutionary processes in nature through experiments, because the evolutionary potential of invasive species is often great (Estoup *et al.* 2016), and may vary between invasive populations (Matesanz *et al.* 2014). This potential could stem from high genetic variation and admixture of repeated introductions (Bock *et al.* 2015; Estoup *et al.* 2016; Roman & Darling 2007) and from more plastic mechanisms or epigenetic processes (Bock *et al.* 2015; Estoup *et al.* 2016). Biological invasions can thus be regarded as natural experiments. They have provided and are continuing to provide insights into ecology and evolution (Lee 2002; Sax *et al.* 2007). In particular, biological invasions are useful to study the temporal dynamics of host–parasite evolutionary interactions in the wild.

### **On how biological invasions can be used as a tool to study host–parasite co-evolution**

Biological invasions represent excellent opportunities to study the ecological and the evolutionary effects of parasites and pathogens (Lafferty *et al.* 2005). Invasions are also ideal opportunities to study co-evolution, because firstly, invasions are experiments performed by nature in a phylogenetically diverse combination of hosts and parasites (Goedknecht *et al.* 2016). Secondly, the time frame of evolutionary changes is often known since it coincides with the time of invasion. It is therefore possible to estimate a rate of change by comparing native, invasive and naïve combinations of host and parasites. Thirdly, several invasion scenarios, in which only the host or the parasite invades, or in which both host and parasite co-invade, lead to different predictions of the underlying ecological and evolutionary dynamics (Figure 1.1; Goedknecht *et al.* 2016). Further, biological invasions are useful to study non-model systems that cannot be kept in the lab for experimental evolution studies, for instance because their long generation times prohibit experiments over multiple

generations. Furthermore, by using biological invasions, it is possible to circumvent the logistical challenges of experimental manipulations on an ecosystem scale to study host–parasite co-evolution in the wild. Here, by making clever use of the natural history of biological invasions of parasites, it is possible to track traits of both host and parasite by performing experiments that contrast both antagonists from different time points along their invasion routes (Feis *et al.* 2016). With this, it is possible to address questions about the adaptive evolution of phenotypes in nature also for those organisms that have longer generation times or cannot be kept in the lab for multiple generations to expose the different generations to each other (time shift experiments).

### **Invasion scenario 1: Invasion of the host**

Invasions by hosts often result in lower parasite burdens (i.e. parasite release or reduction). The lower infection rate and lower parasite burdens relax the selection pressure on the invasive host in its novel range. For those host traits that are costly to express, parasite release upon invasion can cause the loss of these traits, which has been observed frequently (Lahti *et al.* 2009). A shift in selective regimes on the invasive host in its novel range may also release other traits like fecundity or size from evolutionary canalization (Lee 2002), because hosts do not need to allocate energy towards defence against parasites anymore (Maron *et al.* 2004).

Release from parasites can thus reveal the effects of the parasite in the native region (Goedknecht *et al.* 2016; Torchin *et al.* 2003). For example, hosts may reach higher densities, achieve larger body sizes, have a greater biomass (Torchin *et al.* 2001) or an increased dispersal ability (Kelehear *et al.* 2012) in the invaded regions, where they were released from their parasites. In most of these cases, however, it is unclear whether the effects of parasite release just manifest in increased host conditions or also in evolutionary change (e.g. loss of resistance, altered investment into components of fitness other than defence) (Colautti *et al.* 2004). Host–parasite co-evolutionary dynamics are, however, likely to end in this case.

On the other hand, in the invaded region hosts will also be confronted with new native parasites, which they in turn may amplify (i.e., parasite spillback). This process should increase the selection on native hosts, because parasite abundance in the system will increase (Figure 1.1; Goedknecht *et al.* 2016). Furthermore, there will be selection pressure on the invasive host. For instance, invasive populations of Pacific oysters (*Magallana gigas*) evolved resistance to their sympatric bacterial communities

Mechanism	End result	Implication	Invasive host	Native host	Invasive parasite	Native parasite
I Parasite release or reduction		Eco.	Uninfected, hence no direct effects; indirect effects via potential competitive advantage	Direct effects of native parasite; indirect effects via parasite-mediated competition	NA	Potential for host switch to invasive host
		Evo.	Relaxed selection pressure	NA	NA	NA (if diluted see VI)
II Parasite spillback		Eco.	Direct effects of native parasite; indirect effects via apparent competition	Direct effect of native parasite (increased disease risk); indirect effects via apparent competition	NA	Amplification parasite population
		Evo.	Increased selection pressure	Increased selection pressure	NA	NA
III Introduction of free-living stages		Eco.	NA	Direct effect of invasive parasite: emerging disease	Potential to spread in introduced range	NA
		Evo.	NA	Increased selection pressure	Increased selection pressure to infect new potentially less suitable hosts	NA
IV Parasite co-introduction with host		Eco.	Direct effect by co-introduced parasite; indirect effects via parasite-mediated competition	Direct effects of native parasite; indirect effects via parasite-mediated competition	Potential to spread in introduced range	NA
		Evo.	Altered selective landscape due to new genotype by environment interactions of co-invading parasites	NA (if spillover, see V)	Altered selective landscape due to new genotype by environment interactions of co-invading host; with low host density selection for generalism	NA
V Parasite spillover		Eco.	Direct effect of invasive parasite; indirect effects via apparent competition	Direct effect of invasive parasite; indirect effects via apparent competition	High potential to spread in introduced range	NA
		Evo.	NA	Increased selection pressure	Selection for lower host specificity	NA
VI Transmission interference		Eco.	NA	Reduction disease risk by native parasite	NA	Reduced transmission
		Evo.	Adaptation to enemy shifts	Relaxed selection pressure	NA	Selection for lower host specificity to avoid dead ends

**Figure 1.1** Overview of ecological (Eco.) and evolutionary (Evo.) implications of six mechanisms for invasive and native hosts and parasites. I) *Parasite release or reduction*: invasive species can leave all or some of their parasites behind in the native range, leading to potential competitive advantages of invaders over native species (enemy release hypothesis). II) *Parasite spillback*: invasive species can act as new competent hosts for native species, potentially amplifying the native parasite population, leading to increased infection levels in native host species. III) *Introduction of free-living stages of parasites*: invasive species can be parasites themselves, which are introduced as free-living stages (mainly microparasites) in the introduced range. IV) *Parasite co-introduction with host*: invasive species can be co-introduced with their invasive host to the introduced range. V) *Co-introductions of parasites and spillover to native hosts*: invasive parasites can be co-introduced with their invasive host to the introduced range and subsequently spill over to native host species. VI) *Interference with parasite transmission*: invasive species can be non-competent hosts and interfere with the transmission of native parasites. Grey semicircle = invasive host species, white semicircle = native host species, dark grey dot = invasive parasite species, white dot = native parasite species. NA = no available data. Figure and definitions from Goedknecht *et al.* (2016).

Since the time point of invasion was precisely known, the time frame of co-evolution could be estimated to be less than 10 generations (Wendling & Wegner 2015), underlining the suitability of biological invasions of hosts for studying co-evolutionary processes in the wild.

### **Invasion scenario 2: Invasion of the parasite**

Selection on hosts will be amplified during parasite or pathogen invasions. When a parasite invades new areas and infects naïve hosts, there may be unpredictable and highly dynamic trajectories of host and parasite traits (Berenos *et al.* 2011a). Similar to when parasites switch their host species (Longdon *et al.* 2015), it might be difficult to predict co-evolutionary trajectories. The lack of co-evolutionary history can result in strong effects on host populations in parasite outbreak situations, when mass mortalities can also occur. This is especially common in the marine environment (Fey *et al.* 2015). These mass mortalities of infected hosts will not only lead to strong selection on native host populations, but can also result in selection on the invasive parasite, because the host density will be substantially reduced. For instance, the initial mass mortalities of mussels that were associated with the invasion of the intestinal parasitic copepod *Mytilicola intestinalis* (Cole & Savage 1951; Korrynga 1968; Meyer & Mann 1950; Theisen 1966), in combination with low virulence and low mortality rates in contemporary host–parasite combinations, could potentially reflect such an adaptive decrease in virulence, but tests of this hypothesis are lacking.

One evolutionary insight from parasite invasions is that genetic diversity in introduced ranges does not necessarily influence invasion success of parasites, or traits underlying host–parasite interactions (Mineur *et al.* 2014; Weclawski *et al.* 2014; Wielgoss *et al.* 2008). Apart from the many ecological impacts they have for hosts and parasites (reviewed in Goedknecht *et al.* 2016), biological invasions will also alter the fitness landscape of interacting species. Therefore, biological invasions should lead to evolutionary adaptation of both hosts and parasites (Colautti & Barrett 2013). Importantly, when parasite species are translocated (either with their host as a co-introduction or by themselves), their genetic variation is reduced, and on theoretical grounds this bottleneck effect should be amplified for parasites that are co-introduced with their hosts, unless parasite genetic diversity within each introduced host is high. Here, a lower genetic diversity limits the evolutionary potential to adapt to environmental conditions that are encountered in the novel range and that may be



new (Barrett & Schluter 2008), which can influence the invasion success (Roman & Darling 2007). However, even with a bottleneck in genetic variation, sufficient amounts of genetic variation may be present in successful invasions (Facon *et al.* 2006) and successful invasions may also be characterised by high phenotypic plasticity (Lucek *et al.* 2014; Yeh & Price 2004).

Deliberately introduced biocontrol agents provide useful examples of these effects (Lafferty *et al.* 2005) and offer opportunities to study host–parasite co-evolutionary dynamics. Qualitatively similar evolutionary trajectories were observed on two continents after introducing the Myxoma virus to European rabbit populations in Australia and Great Britain (Best & Kerr 2000; Kerr *et al.* 2015; Ross & Sanders 1984), indicating that certain combinations of infectivity/resistance and virulence/tolerance parameters might select for similar (although unpredictable) evolutionary outcomes.

Furthermore, also unintentionally introduced parasites and pathogens can be used to study co-evolutionary dynamics. Selection pressures can differ during the epidemic phase and endemic phase and can lead to rapid changes in virulence (e.g., Knell 2004; Zuckerman *et al.* 2010), infectivity and fitness related traits (e.g., Weclawski *et al.* 2013; Weclawski *et al.* 2014). In general, during the epidemic phase of a new disease, growth rate is expected to be maximised, while during the endemic phase, reproductive output is expected to be maximised (Bull & Ebert 2008). The optimal virulence in the epidemic phase would therefore be higher than in the endemic phase (Frank 1996). Hence, parasite biological invasion can be used to study co-evolutionary processes in nature.

### **Invasion scenario 3: Co-invasion of host and parasite**

When a native combination of host and parasite both invade a new habitat, the host is likely to be the first to expand its range leading to a lag-phase between colonisation of host and parasite (Phillips *et al.* 2010). During this lag-phase, in the absence of selection exerted by the parasite, hosts at the edge of the invasion range can evolve different life history and immune traits, including increased dispersal ability. The same may apply for parasite transmission on its respective range edge, because host density might be low (Kelehear *et al.* 2012). Biological co-invasions can thus demonstrate which traits are under influence of selection for both antagonists.

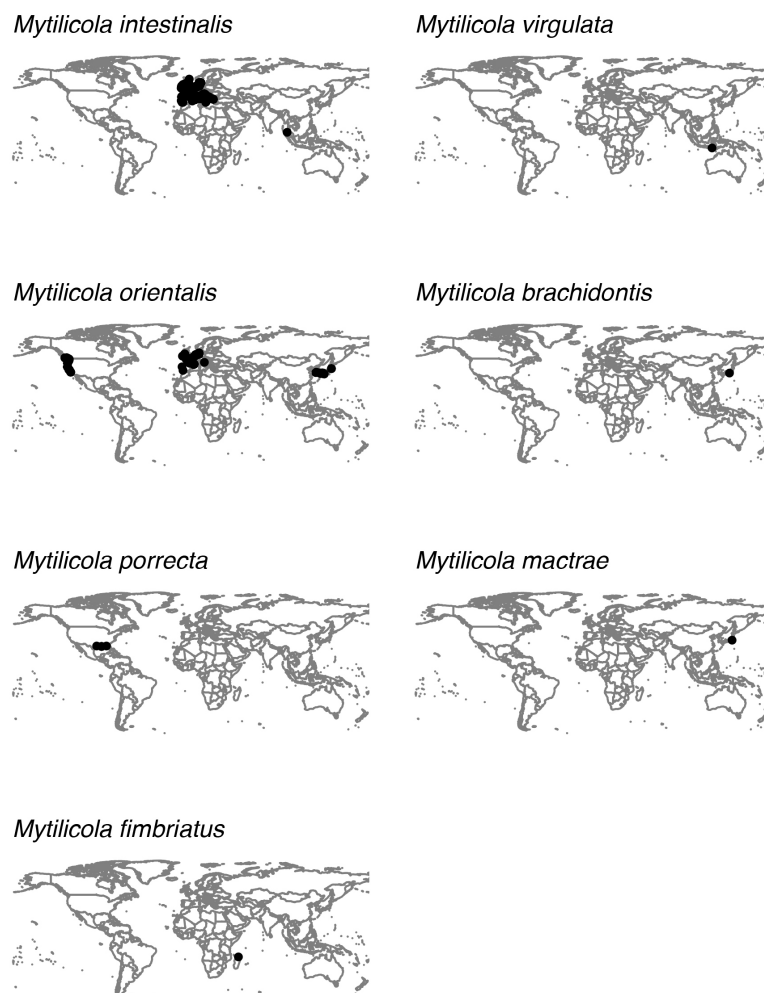
## On the choice of *Mytilicola* spp. as an experimental model

The genus *Mytilicola* belongs to the order of cyclopoid copepods, and all members are parasitic to the intestines of bivalves. Infestation with *Mytilicola* species has been called “mytilicoliasis” (Elston 1993) and “red worm disease”, referring to their appearance. Seven species are currently known from the genus: *Mytilicola intestinalis* Steuer 1902, *Mytilicola virgulata* Leigh-Sharpe 1934 (previously *Piratasta virgulata*), *Mytilicola orientalis* Mori 1935, *Mytilicola brachidontis* Yamaguti 1939 (previously *Piratasta brachidontis*), *Mytilicola porrecta* Humes 1954, *Mytilicola mactrae* Hoshina & Kuwabara 1959 and *Mytilicola fimbriatus* Humes & Ho 1970 (Walter & Boxshall 2018) (Figure 1.2). The life cycle of *Mytilicola* is holoxenous: parasites live in the intestine of the host where they sexually reproduce, and the hatched, free-swimming larvae search for and infest a new host. The life cycle of *M. intestinalis* has been subject of most study (e.g., Gee & Davey 1986b; Grainger 1951; Pesta 1907; Steuer 1905), and the other *Mytilicola* species are assumed to have the same life cycle (Cheng 1967). The life cycle involves the free-living naupliar and free-living metanaupliar stage, the first copepodite stage, which is infective, the second to fifth copepodite stages and adult stages that are fully parasitic (Gee & Davey 1986b; Pesta 1907) (Figure 1.3). Of the seven *Mytilicola* species, *M. intestinalis* and *M. orientalis* have been studied most intensively, owing in part to their (co-)introduction and consequent invasion into novel ranges.

*Mytilicola intestinalis*, the first described species in its genus, was first found in the Adriatic Sea in blue mussels *Mytilus galloprovincialis* (Steuer 1902, 1905) and soon after, also from the Mediterranean Sea, along the French coast in blue mussels *Mytilus edulis* (Vaysi re 1914) and *M. galloprovincialis* (Dollfus 1914). It invaded the North Sea and Atlantic coasts of Europe through ship hull fouling and relaying of infected mussels (reviewed in Korringa 1968). Its invasion was associated with mass mortalities of farmed and wild mussels in the Netherlands and France (Blateau *et al.* 1992; Stock 1950), with associated economic costs from lowered mussel production in both areas (Blateau *et al.* 1992; Drinkwaard 1993).

*Mytilicola orientalis* was described from the Seto Inland Sea in Japan from Pacific oysters *M. gigas* and mussels *Mytilus unguiculatus* (Mori 1935), and it is also known from Korea (Ho & Kim 1992; Kim 2004). It was co-introduced through the transplants of Pacific oysters for aquaculture to the Pacific coast of North America in

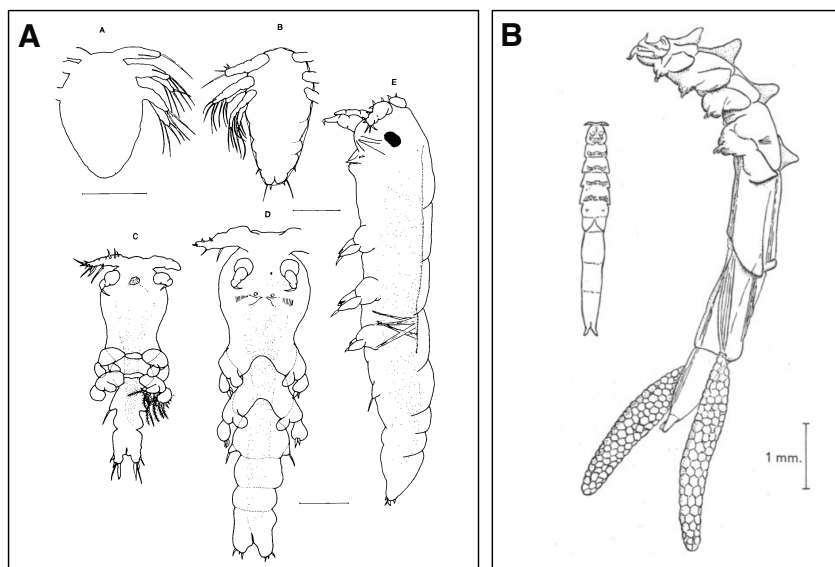
the 1930's (Bradley & Siebert 1978; Odlaug 1946), where it spilled over to native bivalve species, amongst others Olympia oysters *Ostrea lurida* (Odlaug 1946), blue mussels *M. edulis* (Odlaug 1946), mussels *Mytilus californianus* (Chew *et al.* 1964), and the butter clam *Saxidomus gigantea* (Bernard 1969). The invasion of *M. orientalis* in North America led to economic concerns on Pacific oysters and *Ostrea lurida*, because of the lower glycogen content and because oysters with the parasite were not deemed saleable, as they do not look particularly appetizing with “red worms” crawling around (Glude 1975). In the 1970's *M. orientalis* was co-introduced with Pacific oysters into Europe (His 1977b; His *et al.* 1978), where it again spilled over to native bivalve species, e.g., *Mytilus edulis*, common edible cockles *Cerastoderma*



**Figure 1.2** Distributions of *Mytilicola* species. *Mytilicola intestinalis* and *M. orientalis* distributions are based on literature review in Chapter 2; *M. virgulata* from Leigh-Sharpe (1934); *Mytilicola brachidontis* from Yamaguti (1939); *M. porrecta* from Ho & Wardle (1992), Humes (1954), Pearse & Wharton (1938); *M. mactrae* from Hoshina (1959); *M. fimbriatus* from Humes and Ho (1970).

*edule* and Baltic tellins *Limecola balthica* (Goedknecht *et al.* 2017a). In the Mediterranean, *M. orientalis* made it to the list of the Mediterranean's worst 100 invasive species (Streftaris & Zenetos 2006).

The effect of *Mytilicola* species on their hosts has been a topic of debate. It is clear that *Mytilicola* is visibly attached to the gut wall of its host with hooks and obstructs the gut of its hosts (Bignell *et al.* 2008; Figueras *et al.* 1991; Sparks 1962). With histological techniques, it can be seen that the appendages of *Mytilicola* perforate the host's gut wall, causing lesions (Lowe & Moore 1979) with haemocytic infiltration (Santarém *et al.* 1994), causing metaplasia of their hosts' gut epithelium, with a trend towards fibrosis in the areas underneath (His *et al.* 1978; Sparks 1962), and causing ciliated columnar cells of the gut epithelium to be replaced by non-ciliated cuboidal cells (Le Penec & Le Penec 2003). Presence of *M. intestinalis* causes cytoplasmic changes in the hepatopancreas and in the gonads of female mussels, with changes in the number of oocytes that are larger in size, have lysosomes and a different structure of the vitellian membrane (Durfort *et al.* 1982). However, the effect of *M. intestinalis* and *M. orientalis* on the condition (meat content) of infested hosts has not been clear; some authors found a reduction of host condition



**Figure 1.3** *Mytilicola intestinalis*. **A)** Gross morphological characteristics of (a) nauplius; (b) metanauplius; (c) copepodite I (infective stage); (d) copepodite II; (e) copepodite III. Ventral views, except (e), which is a lateral view. Scale bars = 0.1 mm. **B)** *Mytilicola intestinalis*, adult male (left) from the ventral aspect, and female (right) with egg-sacks in left dorso-ventral view. The dorso-lateral processes are shown, by which the animal presses the ventral surface with appendages against the opposite wall of the hosts's intestine. Figure 1.3A including figure captions taken from Geeand Davey (1986b) and Figure 1.3B including figure captions taken from Hockley (1951).

(e.g., Cole & Savage 1951; Katkansky *et al.* 1967; Odlaug 1946), and lower in the glycogen content of infested hosts (Glude 1975), while others did not find a reduction in condition (e.g., Bernard 1968; De Grave *et al.* 1995; Dethlefsen 1975; Steele & Mulcahy 2001) or glycogen levels (Moore *et al.* 1977), although it must be noted that most of these observations are based on correlational field studies and not on experimental evidence.

The natural history and the invasion of *M. intestinalis* therefore make it an excellent model organism to use for studying host–parasite co-evolution in the wild. The anecdotal invasion routes of *M. intestinalis* have been well described within the Wadden Sea, where it first invaded around the Bremerhaven–Cuxhaven area (Caspers 1939), from which it spread southwest toward the island of Texel (the Netherlands) (Drinkwaard 1993) and north towards the island of Sylt (Germany) (Dethlefsen 1972), arriving at both islands in the early 1970’s. The hosts and parasites there thus have a similar amount of co-evolutionary time, which sets the stage for studying them. Furthermore, both the host *M. edulis* as well as *M. intestinalis* can be brought into the lab for infection experiments (Gee & Davey 1986a; Hepper 1953), and an anti-*Mytilicola* treatment for mussels exists (Blateau *et al.* 1992), which makes it possible to take mussels and parasites from infected mussel beds to carry out local adaptation experiments, contrasting sympatric versus allopatric host–parasite combinations. In experiments, pre-infection traits (infectivity and resistance) can be separated from post-infection traits (virulence and tolerance), because the number of copepodites can be counted before infection, and the parasite is not able to reproduce clonally. Furthermore, upon invasion in Europe and the Wadden Sea, *M. intestinalis* and *M. orientalis* now share the same range and the same host: *M. edulis*. While *M. intestinalis* utilises only *M. edulis* as a host in the Wadden Sea, *M. orientalis* also utilises other hosts (Goedknecht *et al.* 2017a). This sets up the perfect conditions for testing whether there is competition between the two species sharing the same host, and if there is a cost to generalism.

## **Thesis aims and objectives**

The aim of this thesis was to study host–parasite co-evolution and co-infection in the wild. To meet this aim, I used the biological invasions of the parasitic copepods

*Mytilicola intestinalis* and *Mytilicola orientalis* in the Wadden Sea as tools. The four objectives of this thesis were (i) to validate the anecdotal origins and invasion routes of the two invasive copepods *M. intestinalis* and *M. orientalis* (Chapter 2), (ii) to test whether there is competitive superiority between two invasive parasitic copepods *M. intestinalis* and *M. orientalis* that share the same novel host, *M. edulis*, but that have different degrees of host specificity in the Wadden Sea (Chapter 3), (iii) to describe the co-evolutionary trajectories on a phenotypic level for the native host *Mytilus edulis* and the invasive parasite *M. intestinalis* after its invasion in relation to naïve hosts lacking co-evolutionary interactions (Chapter 4), (iv) to find out the molecular mechanisms behind the different host–parasite interactions that were found at the separate invasion fronts in Chapter 4 (Chapter 5).

## Chapter 2

### **Global invasion genetics of two parasitic copepods infecting marine bivalves**

The invasions and impacts of the two marine parasites *M. intestinalis* and *M. orientalis* have been studied extensively because of their potential economic threats to the mussel and oyster aquaculture. However, validation of the native ranges and invasion routes of both parasites is still lacking. Accordingly, it was my aim to confirm the anecdotal invasions routes acquired through an extensive literature search with invasion genetics methods of the mitochondrial COI gene of samples from the putative native regions and the invaded regions.

## Chapter 3

### **Do invasions create competitors? Co-infections of specialist *Mytilicola intestinalis* vs. generalist *Mytilicola orientalis* in blue mussel hosts**

Specialist parasites should have a competitive advantage over generalists due to costs of generalism. Generalist and specialist taxa should therefore not occupy the same niche over extended periods of time. However, biological invasions can create new competitive situations and in this chapter, I used the consecutive invasions of two congeneric parasitic copepods to test the competitive balance between generalist and specialist shortly after invasion. My major aim was to elucidate whether competitive ability of generalist and specialist parasites contribute to their invasion success. To meet this aim, I test whether there is superiority of specialist *M. intestinalis* over

generalist *M. orientalis* in their shared host, and if there is a cumulative effect of co-infections on the condition of their shared host at similar total parasite loads.

#### *Chapter 4*

### **Biological invasions and host–parasite co-evolution: different co-evolutionary trajectories along separate parasite invasion fronts**

Theoretical predictions of the dynamics underlying host–parasite co-evolution have largely been confirmed in experimental evolutionary systems. However, proof-of-principle in natural systems has rarely been made, and has largely focused on microparasites, in which it is difficult to separate infectivity from virulence. The objective of this chapter is to show that biological invasions are a great tool to study host–parasite co-evolution in the wild. I took an experimental approach to study the phenotypic traits of infection by making naïve, sympatric and allopatric host–parasite infection combinations. I looked at whether the invasive parasite *M. intestinalis* or the native host *M. edulis* are locally adapted, and if phenotypic traits of host (resistance or tolerance) or parasite (infectivity or virulence) had similar evolutionary trajectories at different invasion fronts in the Wadden Sea.

#### *Chapter 5*

### **Dual transcriptomics reveals co-evolutionary mechanisms of intestinal parasite infections in blue mussels *Mytilus edulis***

The underlying molecular mechanisms and evolutionary steps of the antagonistic co-evolution between hosts and their parasites remain elusive, especially in non-model systems. My aim was to characterise the host–parasite interactions on a transcriptional level to shed light on the underlying molecular mechanisms behind the different evolutionary trajectories of mussel and *Mytilicola* found in *Chapter 4*. I chose to study phenotypes instead of genetic composition because selection acts on phenotypes. Hereto, I applied a novel dual-species transcriptomics approach to experimental cross-infections of blue mussels *M. edulis* and their invasive parasitic copepod *M. intestinalis*.





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Global invasion genetics of two parasitic copepods  
infecting marine bivalves

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## Abstract

Invasive species, and especially invasive parasites, are great tools to study ecology and evolution in the wild. The consecutive invasions of the two parasitic copepods *Mytilicola intestinalis* and *Mytilicola orientalis* have been proven to be particularly useful to study ecological consequences and coevolution with their newly acquired host *Mytilus edulis* in the North Sea. To fully understand these processes a detailed knowledge of the invasion history and invasion routes from their respective native ranges is necessary. Therefore, we now combine an extensive literature survey with a global phylogeography of partial COI sequences that covers the reported putative native regions (i.e., Japan for *M. orientalis* and the Mediterranean Sea for *M. intestinalis*) as well as the present day invaded regions (North America and European Atlantic and North Sea coasts) of both species. The population genetic analyses confirmed the native region and invasion route of *M. orientalis*: there was significant population differentiation between the native regions in the Seto Inland Sea and the Sea of Japan, while populations in North America and Europe could not be distinguished from the Seto Inland Sea locations resembling the genetic structure and recent spread of the parasite's principle host, the Pacific oyster *Magallana gigas*. This suggests that *M. orientalis* is native in Japan and invasive in North America and Europe, confirming the reported invasion history. *Mytilicola intestinalis*, on the other hand, showed a lack of population genetic structure coupled to low genetic diversity in the putative native regions as well as in the invaded regions. Therefore, the native origins of *M. intestinalis* remain unclear. Our study shows that even though invasive species can be studied extensively in the past, basic knowledge on their native range and invasion history can still lack.

*Keywords:* macroparasite; invertebrates; invasion genetics; mtDNA; genetic diversity; cryptogenic species

## Introduction

There is a tremendous amount of invasive species today, which poses a great threat to global natural ecosystems, biodiversity, agriculture, economy and human health (Early *et al.* 2016; Millennium Ecosystem Assessment 2005; Pimentel *et al.* 2001). Invasion rates have accelerated in the last decades (e.g., Cohen & Carlton 1998; Jackson & Grey 2012), and numbers of invasive species are predicted to increase further with increasing international trade (Levine & D'Antonio 2003). Biological invasions can alter ecosystem processes (Ehrensfield 2010) and have an ecological and evolutionary impact in their novel ranges (e.g., Gallardo *et al.* 2015; Mooney & Cleland 2001; Reaser *et al.* 2007; Suarez & Tsutsui 2008). If invasive species are parasites, invasions can have wide-ranging effects when they are introduced to new ecosystems as free-living stages or when they are co-introduced with a host and can spill over to native host species, which are mechanisms with strong ecological and evolutionary implications (Goedknecht *et al.* 2016). Therefore, understanding origins and pathways of invasive parasites is important. In this context, the traditional way of using literature records can be fruitfully complemented by the use of population genetic analyses. Phylogeographic studies of species in their putative native and invaded regions (“invasion genetics”, see Barrett 2015) can provide more detail to clarify their origin, invasion routes, population genetic structure and genetic diversity (Cristescu 2015). However, invasive parasites are unfortunately understudied in this respect, as invasive parasites in general are understudied (Poulin 2017).

Two exceptions to this general rule are the consecutive invasions of the two parasitic copepods *Mytilicola intestinalis* and *Mytilicola orientalis* that have been proven to be particularly useful to study ecological consequences and coevolution with their newly acquired blue mussel host (*Mytilus edulis*) along European Atlantic shores. *Mytilicola intestinalis* invaded European waters in the early 20<sup>th</sup> century through ship hull fouling and relaying of infected mussels (e.g., Caspers 1939; Ellenby 1947; Korrynga 1968). This invasion was recently used as a tool to study host–parasite co-evolution in the wild: by cross-infecting hosts and parasites from separate invasion fronts with similar amounts of co-evolutionary time, Feis *et al.* (2016) (*Chapter 4*) could show that parasite invasion led to different evolutionary trajectories that are characterised by specific molecular interactions between host and parasite (Feis *et al.* 2018) (*Chapter 5*). *Mytilicola orientalis* was co-introduced with

invasive Pacific oyster *Magallana gigas* through transplants for aquaculture (His 1977b; Odlaug 1946) and via spillover events, now also infects native hosts including the mussel *M. edulis* (Chew *et al.* 1964; Goedknecht *et al.* 2017a; Odlaug 1946). Thus, *M. edulis* is now a host shared between *M. intestinalis* and *M. orientalis*.

The reported invasion histories already suggest substantial differences in the mode of invasion of these two *Mytilicola* species that could in turn feed back onto the ecological effects and evolutionary responses in the newly invaded shared host. While the major invasion routes of the Pacific oyster are clear (Moehler *et al.* 2011; Rohfritsch *et al.* 2013), the invasion route of its hitchhiking parasite *M. orientalis* has yet to be validated. Similarly, the invasion routes of *Mytilus galloprovincialis*, the proposed native host of *M. intestinalis*, are known better than those of its parasitic copepod *M. intestinalis* (Pickett & David 2018; Śmietanka *et al.* 2014). To further understand the invasion history and consequences for ecological and evolutionary processes, the genetic structure in the native ranges and along invasion routes needs to be determined first.

Therefore, our aim with this paper was 1) to use the available literature to identify putative native regions and reconstruct the invasion routes of both parasites, against which we could formulate specific hypotheses of what genetic structure should look like, and 2) to test whether the areas of origin of *M. intestinalis* and *M. orientalis* are the Mediterranean Sea and Japan, respectively, and 3) within those putative native regions, to determine a more specific location from which both species may have invaded. We do so through sequencing part of the cytochrome-*c*-oxidase I (COI) gene, and testing the genetic diversity and population differentiation between putative native and known invaded regions for both invasive *Mytilicola* species.

## **Materials and methods**

### **Literature review for plotting occurrence maps**

To describe the species distribution through time in putative native and invaded regions of both invasive *Mytilicola* species, we searched the Web of Science/Web of Knowledge and Google Scholar with the keywords “*Mytilicola intestinalis*” and “*Mytilicola orientalis*”. Further publications were found by checking the references of each relevant publication (i.e., “snowballing”). All publications were screened for

records of number of hosts dissected, prevalence and infection intensity of the respective *Mytilicola* species per host species, and for coordinates of sampling sites. This resulted in data tables for *Mytilicola intestinalis* and for *Mytilicola orientalis* (available upon request), which were used for plotting occurrence maps. If latitude and longitude were not reported in a publication, we inferred approximate coordinates with Google maps so that the data could be plotted.

### **Sampling of host and parasite species**

Mussels *Mytilus edulis*, *Mytilus galloprovincialis*, *Mytilus trossulus* and *Brachidontes pharaonis* and Pacific oysters *Magallana gigas* were collected by hand in the intertidal zone during low tide at 61 locations along the Mediterranean, the Atlantic and the North Sea coasts of Europe, in the Baltic Sea, at the Pacific coasts of the United States of America and Canada, along the coasts of the Seto Inland Sea, in the Sea of Japan and in the Oyashio Current (Table 2.1). The bivalves were dissected in the lab under a dissection microscope or with the help of a magnifying glass in order to screen for macroparasites. *Mytilicola* spp. specimens were isolated and stored in 100% ethanol.

We report parasite prevalence as the number of individual mussels or oysters infected with *M. intestinalis* or *M. orientalis* divided by the total number of mussels or oysters examined per location, and mean intensity as the mean number of *M. intestinalis* or *M. orientalis* within an infected mussel or oyster per location (*sensu* Margolis *et al.* 1982). Part of the *M. orientalis* sample from Tavira (Portugal) was acquired through an anti-*Mytilicola* treatment of mussels using Dichlorvos (Blateau *et al.* 1992; Feis *et al.* 2016). Fifteen live *Mytilicola* specimens escaped the mussels during and shortly after the treatment. Prevalence and mean intensity (Table 2.1) for Tavira were calculated based on dissected mussels only.

### **DNA extraction, amplification and sequencing**

DNA was extracted from individual *Mytilicola* using either the QIAgen 96 DNeasy Blood & Tissue kit or the Zymo Research genomic DNA kit (the latter by Baseclear B.V., Leiden, the Netherlands). All extractions were performed according to the protocols provided by the manufacturers. DNA concentration was measured on a Nanodrop to confirm DNA quality and quantity. A 534 basepair (bp) fragment of the mitochondrial cytochrome-*c*-oxidase I region (COI) was amplified using the primer

pair MOICOIf and MOICOIr for *M. intestinalis* (Goedknecht *et al.* 2018) and a newly developed primer pair for *M. orientalis* (MoriCOI50f: 5'-TTG ATC GGG CTT AAT TGG-3' and MoriCOI50r: 5'- GAT CGG TTA ARA GCA TGG T -3'). The *M. orientalis* primers were developed in CLC Genomics Workbench 8.5.1 (CLCbio, Denmark, <https://www.qiagenbioinformatics.com/>) and were based on *M. orientalis* COI sequences from Elsner *et al.* (2010).

For *M. intestinalis*, polymerase chain reaction (PCR) and Sanger sequencing using both forward and reverse primer were performed according to Goedknecht *et al.* (2018) at Baseclear B.V. (Leiden, Netherlands). For *M. orientalis*, PCR was performed with Hotstart kit in 30 µl reaction volumes, adding 15 µl Hotstart Mastermix, 5 µl of each primer, 11 µl H<sub>2</sub>O and 1 µl of sample for each reaction. Amplification started with a heat-activation of 15 min at 95 °C, followed by 38 cycles of 95 °C for 30 s, annealing at 50 °C for 30 s and elongation at 72 °C for 60 s. The final extension step was at 72 °C for 10 min. Amplification success of all *M. orientalis* samples were checked with QIAxcel (QIAGEN, Hilden, Germany). PCR purification and Sanger sequencing of *M. orientalis* samples were performed on both the forward and reverse strand at the Institute of Clinical Molecular Biology (Kiel, Germany).

### **Data analyses**

Quality control of the sequences was performed in CLC Genomics Workbench 8.5.1 (for *M. orientalis*) or BioEdit 7.2.5 (for *M. intestinalis*) (Hall 1999), after which forward and reverse sequences were combined. We screened all data for double peaks and at least one of the forward and reverse sequences had no double peaks at any base in the sequence. Some of the North American samples failed to amplify or sequence entirely and were not considered. COI sequences were cropped to 483 base pairs (bp) for *M. intestinalis* and to 476 bp for *M. orientalis*. Haplotypes were found using DnaSP 5 (*M. orientalis*) (Librado & Rozas 2009) or using custom Python 3.6 script (*M. intestinalis*). Arlequin 3.5.1.2 (Excoffier & Lischer 2010) was used to calculate population comparisons (10,000 permutations), population differentiation (10,000 permutations), Analyses of MOlecular VARIance (AMOVA), molecular diversity indices, Tajima's D statistic (Tajima 1989) and to perform Fu's F<sub>s</sub> tests (Fu 1997; Fu & Li 1993) for selective neutrality (10,000 simulated samples), mismatch distributions (10,000 permutations) and minimum spanning networks for both species.

For neutral markers, Tajima's  $D$  and Fu's  $F_s$  can be used to detect changes in population size. Significant negative  $D$  and  $F_s$  values can be interpreted as signatures of population expansion or, unless another independent marker has the same pattern, a past selective sweep (Fu 1997; Fu & Li 1993). In our analyses we only included locations with more than 15 samples, with the exception of calculations for minimum spanning networks, in which all samples were included. In addition to overall AMOVA, we also calculated AMOVAs within putative native and introduced regions for both species, and hierarchical AMOVAs for Mediterranean *M. intestinalis* with two designs to test whether the overall population differentiation can be attributed to different regions within the Mediterranean Sea. The first design tested the Gulf of Lion (Cassis, Istres, Rouet and Bouzigues) vs. the Ligurian Sea (Livorno) vs. the Adriatic Sea (Malinska and Marjan). In the second design, the location Cassis was added to the Ligurian Sea group instead of the Gulf of Lion group. To test for differences in genetic diversity between species and regions, we fitted a linear model testing haplotype diversity ( $h$ ) and nucleotide diversity ( $\pi$ ) as a function of parasite species (levels: *M. intestinalis* and *M. orientalis*), region (levels: putative native region, introduced region) and the interaction term parasite species  $\times$  region in the R statistical environment version 3.4.3 (R Core Team 2017). Pie charts representing haplotype frequencies per location were also made in R. Maps were made with the R packages rworldmap (South 2011) and ggplot2 (Wickham 2009).

## Results

### Literature

A search for "Mytilicola intestinalis" resulted in 72 hits on Web of Science/Web of Knowledge (all databases) and 934 hits on Google Scholar (15 December 2017). A search for "Mytilicola orientalis" resulted in 15 hits on Web of Science/Web of Knowledge (all databases), and 373 hits on Google Scholar (15 December 2017). More literature was found by snowballing. Our literature survey led to the inclusion of 172 publications on occurrences of *M. intestinalis* (2187 data points including those

**Table 2.1** Parasitic copepods *Mytilicola intestinalis* (MI) and *M. orientalis* (MO) collected from mussels: *Mytilus galloprovincialis* (Mg), *Mytilus edulis* (Me), *Mytilus trossulus* (Mt) and *Brachidontes pharaonis* (Bp), and from oysters: Pacific oysters *Magallana gigas*. All hosts were collected in harbours or on natural mussel beds, which were almost all situated in intertidal areas (unless indicated).  $N_{\text{host}}$ : total number of hosts dissected;  $N_{\text{parasites}}$ : number of parasites successfully sequenced for cytochrome-c-oxidase 1; Collectors and dissectors: collectors are indicated in italics, dissectors by underlining. Note that no *M. intestinalis* was found in Pacific oysters in this study, therefore the column representing this host-parasite combination was omitted from the table.

Location	Country	Coordinates	Year	$N_{\text{hosts}}$		Prevalence (%)			Mean infection intensity			$N_{\text{parasites}}$		Collectors and Dissectors	
				mussel	oyster	MI mussel	MO mussel	MO oyster	MI mussel	MO mussel	MO oyster	MI	MO		
<b>Seto Inland Sea, Sea of Japan and Oyashio Current</b>															
Iwaya, Fukuoka	JPN	33°55'58" N, 130°41'08" E	2015	15 Mg	287	0.0	0.0	5.2	-	-	1.8	-	24	<i>S. Hashimoto</i> , <u>YK</u> , <u>MEF</u>	
Ayaragi, Yamaguchi	JPN	34°0'37" N, 130°54'57" E	2015	0	16	-	-	0.0	-	-	-	-	-	<i>S. Hashimoto</i> , <u>YK</u> , <u>MEF</u>	
Ube harbour, Yamaguchi	JPN	33°56'33" N, 131°14'44" E	2015	0	150	-	-	0.0	-	-	-	-	-	<i>S. Hashimoto</i> , <u>YK</u> , <u>MEF</u>	
Ondo-no-Seto, Hiroshima	JPN	34°11'35" N, 132°32'05" E	2015	44 Mg	305	0.0	2.3	10.1	-	1.0	1.4	-	33	<u>YK</u> , <u>MEF</u>	
Kamo River bridge, Takehara, Hiroshima	JPN	34°19'41" N, 132°53'52" E	2015	0	175	-	-	10.3	-	-	2.4	-	29	<i>H. Uchiyama</i> , <u>YK</u> , <u>SO</u> , <u>MEF</u>	
Mukaishima, Hiroshima	JPN	34°22'59" N, 133°10'22" E	2015	0	26	-	-	0.0	-	-	-	-	-	<i>M. Urata</i> , <u>MEF</u>	
Niihama, Ehime	JPN	33°58'56" N, 133°17'37" E	2015	0	265	-	-	10.6	-	-	1.8	-	30	<i>S. Hashimoto</i> , <u>YK</u> , <u>MEF</u>	
Tarumi Bay, Kagawa	JPN	34°22'41" N, 133°56'42" E	2015	0	300	-	-	6.3	-	-	1.3	-	21	<i>S. Hashimoto</i> , <u>YK</u> , <u>MEF</u>	
Hiwasa, Tokushima (location 1)	JPN	33°43'47" N, 134°31'59" E	2015	0	100	-	-	0.0	-	-	-	-	-	<i>S. Hashimoto</i> , <u>YK</u> , <u>MEF</u>	
Hiwasa, Tokushima (location 2)	JPN	33°44'33" N, 134°33'23" E	2015	0	115	-	-	0.8	-	-	1	-	1	<i>S. Hashimoto</i> , <u>YK</u> , <u>MEF</u>	
Ofunato, Iwate	JPN	39°03'19" N, 141°43'27" E	2015	0	125	-	-	1.6	-	-	1	-	1	<u>YK</u> , <u>MEF</u>	
Onizawa, Iwate	JPN	39°05'14" N, 141°48'42" E	2015	0	76	-	-	1.3	-	-	1	-	-	<u>YK</u> , <u>MEF</u>	
Sakihama, Iwate	JPN	39°05'56" N, 141°51'36" E	2015	0	100	-	-	1.0	-	-	1	-	-	<u>YK</u> , <u>MEF</u>	
				<b>59 Mg</b>	<b>2040</b>							<b>0</b>	<b>139</b>		
<b>Pacific coast of North America</b>															
Belfair State Park, Hood Channel	USA	47°25'45" N, 122°52'24" W	2015	11 Mt	55	0.0	0.0	10.9	-	-	5.8	-	22	<u>KMW</u> , <u>LNSS</u>	
Harstine Island	USA	47°14'58" N, 122°52'14" W	2015	20 Mt	20	0.0	0.0	5.0	-	-	1.0	-	1	<u>KMW</u> , <u>LNSS</u>	
Grappler inlet, Bamfield	CAN	48°49'45" N, 125°7'40" W	2015	23 Mt	0	0.0	4.3	-	-	1.0	-	-	0	<u>KMW</u> , <u>LNSS</u>	
Piper's Lagoon, Nanaimo	CAN	49°13'34" N, 123°56'59" W	2015	20 Mt	64	0.0	25.0	29.7	-	1.6	1.6	-	5	<u>KMW</u> , <u>LNSS</u>	
Fanny Bay	CAN	49°30'23" N, 124°49'35" W	2015	20 Mt	0	0.0	20.0	-	-	2.3	-	-	2	<u>KMW</u> , <u>LNSS</u>	
Morning Beach, Denman Island	CAN	49°35'56" N, 124°49'31" W	2015	0	49	-	-	14.3	-	-	2.3	-	0	<u>KMW</u> , <u>LNSS</u>	
Manson's Landing, Cortes	CAN	50°4'17" N, 124°58'50" W	2015	10 Mt	30	0.0	60	6.7	-	6.0	1.0	-	8	<u>KMW</u> , <u>LNSS</u>	
				<b>104 Mt</b>	<b>218</b>							<b>0</b>	<b>38</b>		
<b>Mediterranean Sea and Adriatic Sea</b>															
Bouzigues	FRA	43°26'51" N, 3°39'06" E	2016	110 Mg	58	20.0	0.0	1.7	1.3	-	2.0	30	-	<u>PCL</u> , <u>MEF</u>	





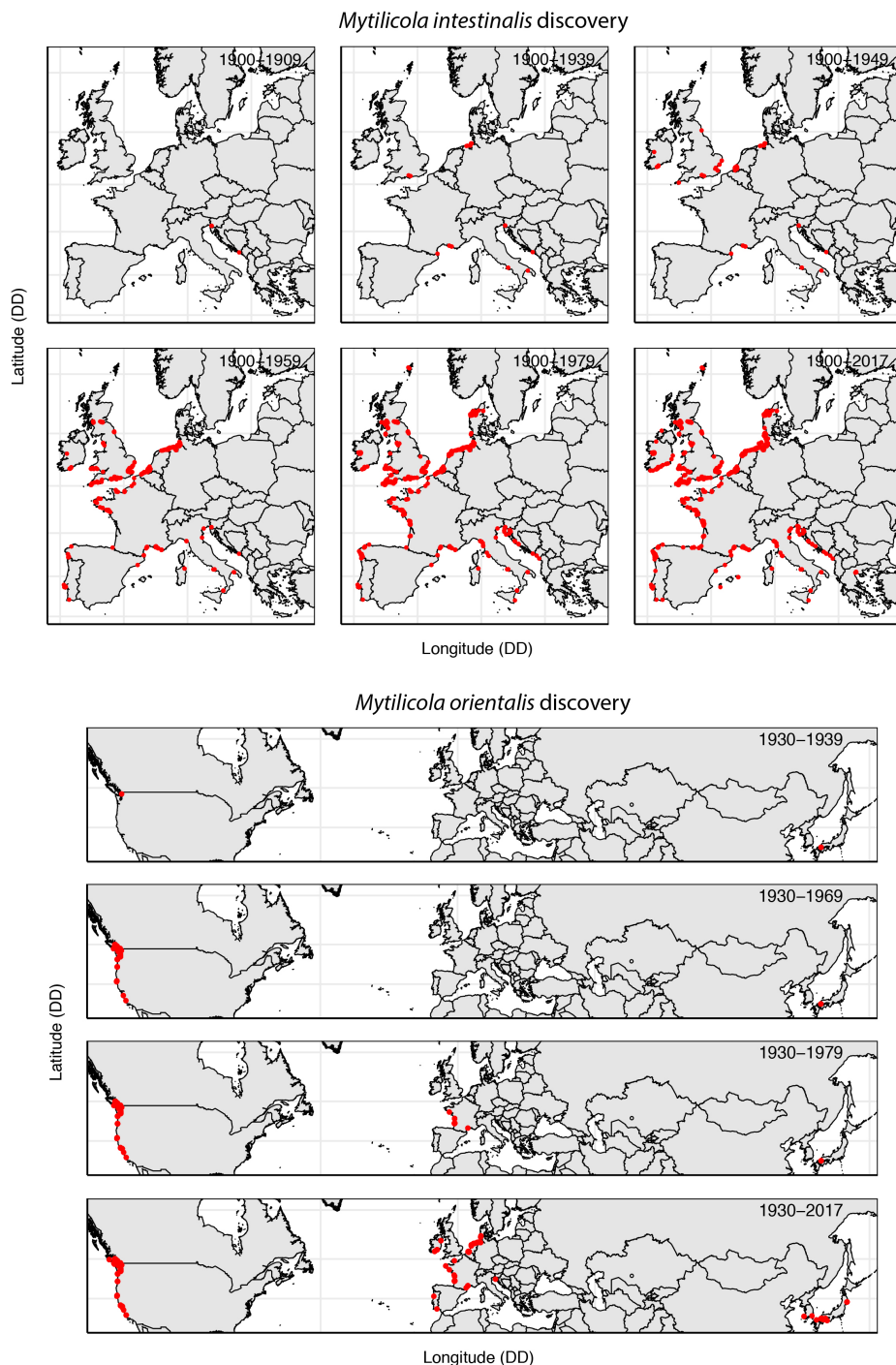
Puan Klent, Sylt	DEU	54°47'54" N, 08°18'11" E	2012	80 Me	80	93.8	60	17.5	4.0	2.6	4.6	30	25	R. Nautia, MAG
Königshafen, Sylt	DEU	55°01'45" N, 8°26'03" E	2012	80 Me	80	78.8	1.3	0.0	3.2	1.0	-	23	-	R. Nautia, MAG
Helgoland (subtidal)	DEU	54°10'31" N, 7°53'41" E	2014	150 Me	0	0.0	0.0	-	-	-	-	-	-	AWI divers, MEF
Helgoland	DEU	54°10'45" N, 7°53'22" E	2014	0	20	-	-	0.0	-	-	-	-	-	MEF
<b>Region total</b>				<b>790 Me</b>	<b>660</b>							<b>177</b>	<b>204</b>	
<b>Baltic Sea</b>														
Kiel Bülk	DEU	54°27'15" N, 10°11'53" E	2014	75 Me	0	0.0	0.0	-	-	-	-	-	-	A. Pansch, MEF
Kiel Fjord	DEU	54°19'49" N, 10°08'59" E	2014	115 Me	0	0.0	0.0	-	-	-	-	-	-	A. Pansch, MEF
<b>Region total</b>				<b>190 Me</b>	<b>0</b>							<b>0</b>	<b>0</b>	
				<b>2658 Mg</b>										
<b>TOTAL ALL LOCATIONS</b>				<b>&gt; 1595 Me</b>	<b>5604</b>							<b>424</b>	<b>444</b>	
				<b>104 Mt</b>										
				<b>90 Bp</b>										

of this study; references in the Supplementary materials) and of 47 publications on occurrences of *M. orientalis* (416 data points including those of this study; references in the Supplementary materials). The literature included peer-reviewed publications, fishery leaflets, reports, book sections and dissertations. A total of 41 publications with potentially relevant information could not be viewed because the full texts were not available online, and could also not be accessed via Dutch and German national libraries, or by attempts to contact the original authors, and could thus not be included in the tables.

The distribution of *Mytilicola intestinalis* in the early 20<sup>th</sup> century was only in the Mediterranean Sea, where it was recorded from a few locations near large ports and aquacultural areas (Figure 2.1, Figure S2.1). The hypothesised native region of *M. intestinalis* is therefore the Mediterranean Sea. Because of the long geographic history of the Mediterranean Sea, its different hydrographic regimes and the short larval phase of *M. intestinalis*, we expect that *M. intestinalis* has a high genetic diversity in this region and that there is population genetic structure between the different basins. In the 1930's its distribution spread and included the German and English coasts at a few specific locations, from which, in the following years, the distribution spread further. In the late 1940's the parasite was first observed in the Netherlands and Ireland, followed by France in the early 1950's. In the 1950's there was a concerted effort to record the invasion and potential effects of *M. intestinalis* along the Atlantic coasts of Europe, which resulted in the publication of a special issue in *Revue des Travaux de l'Office Scientifique et Technique des Pêches Maritimes* on *M. intestinalis* (Le Gall 1951). Ship hull fouling was seen as the main vector of *M. intestinalis* introductions, after which further spread was aided by relaying of mussels (i.e., German Wadden Sea to aquaculture areas in Zeeland, the Netherlands). It is thus unclear from which Mediterranean basin (or basins) *M. intestinalis* was introduced, and whether this happened repeatedly, which might lead to different expectations regarding signatures of bottlenecks and genetic diversity in the introduced region.

The distribution of *M. orientalis* in its putative native region, Japan and Korea, has not been the subject of many studies, which is reflected in our literature survey (Figure 2.1, Figure S2.2). We expect high genetic diversity in the native region, as well as population genetic differentiation. The co-introduction of *M. orientalis* with the Pacific oyster in North America has been much better recorded. In the 1970's, Pacific oysters were imported into France, and, once more, *M. orientalis* was co-

introduced across a long distance. Soon after that it was also found in Ireland via transported oysters. There are many gaps in the distribution of *M. orientalis* in



**Figure 2.1** Temporal development of the invasions of *Mytilicola intestinalis* and *Mytilicola orientalis*. Locations where *Mytilicola* spp. presence has been recorded are indicated with red dots, showing the invasion route and spread, as well as research effort through the years. For temporal development of *M. intestinalis* and *M. orientalis* decade-by-decade, including species absence records, see Figures S2.1 and S2.2 respectively. This figure is based on data from this study and from data acquired during our literature surveys. See the Supplementary materials for references.

Europe, which presumably reflect the lack of studies in those areas (Figure S2.2). Because of the many transports of Pacific oysters to North America and Europe, we do not expect to find a reduction in *M. orientalis* genetic diversity in the invaded regions.

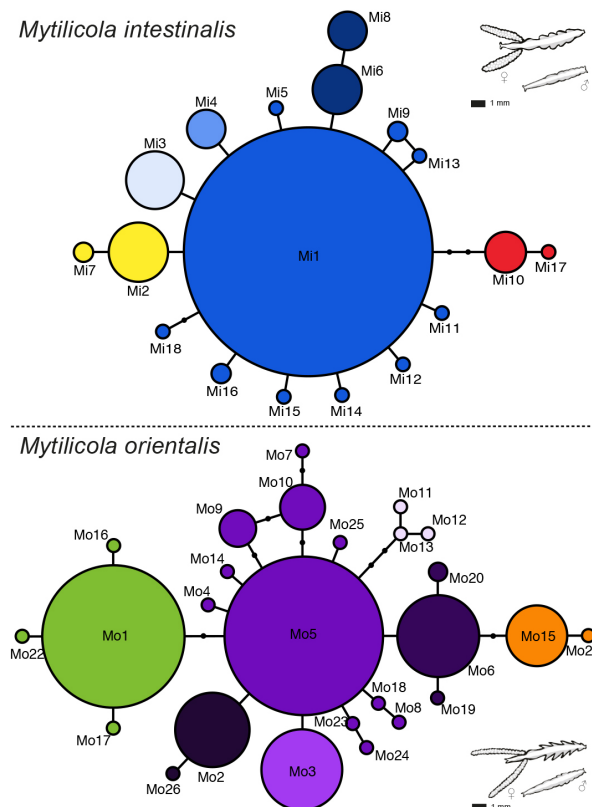
### **New records of *Mytilicola* spp.**

A total of 5604 Pacific oysters *Magallana gigas*, 2658 mussels *Mytilus galloprovincialis*, more than 1595 mussels *Mytilus edulis*, 104 mussels *Mytilus trossulus* and 90 mussels *Brachidontes pharaonis* were dissected in this study. Dissections of Pacific oysters and mussels from 20 locations along Mediterranean and Adriatic coasts, 13 locations in Japan, 7 locations along the west coast of North America, and 21 locations along the northeastern Atlantic, North Sea, Wadden Sea and Baltic coasts of Europe resulted in detection of *M. intestinalis* at 20 locations and a detection of *M. orientalis* at 30. Of the locations we sampled in Japan, the only locations where *M. orientalis* had been previously recorded were Takehara in the Hiroshima prefecture (Nagasawa & Nitta 2014), and Hiwasa in the Tokushima prefecture (Do & Kajihara 1986) – the other locations, including those outside the Seto Inland Sea, are thus new records for *M. orientalis*. In addition, Tavira (Portugal) can be added to this list. The Mediterranean locations Malinska, Marjan (both Croatia) and Cassis (France) are new records for *M. intestinalis*, although Cassis is close to locations *M. intestinalis* was already known from (i.e., Berre Lagoon and Marseille). *Mytilicola intestinalis* was detected in *M. galloprovincialis* and *M. edulis*, while *M. orientalis* was detected in *M. gigas* and *M. trossulus* on top of that. Prevalences of *M. intestinalis* ranged from 0 to 82.0% in the Mediterranean and Adriatic, the putative native region of *M. intestinalis*, and from 0 to 93.8% in the invaded regions (Table 2.1). Prevalences of *M. orientalis* ranged from 0 to 10.6% in Japan, the putative native region of *M. orientalis*, and from 0 to 73.8% in the invaded regions (Table 2.1). A total of 424 *M. intestinalis* and 444 *M. orientalis* were successfully sequenced, which resulted in the detection of 18 and 26 haplotypes, respectively (Tables S1 and S2).

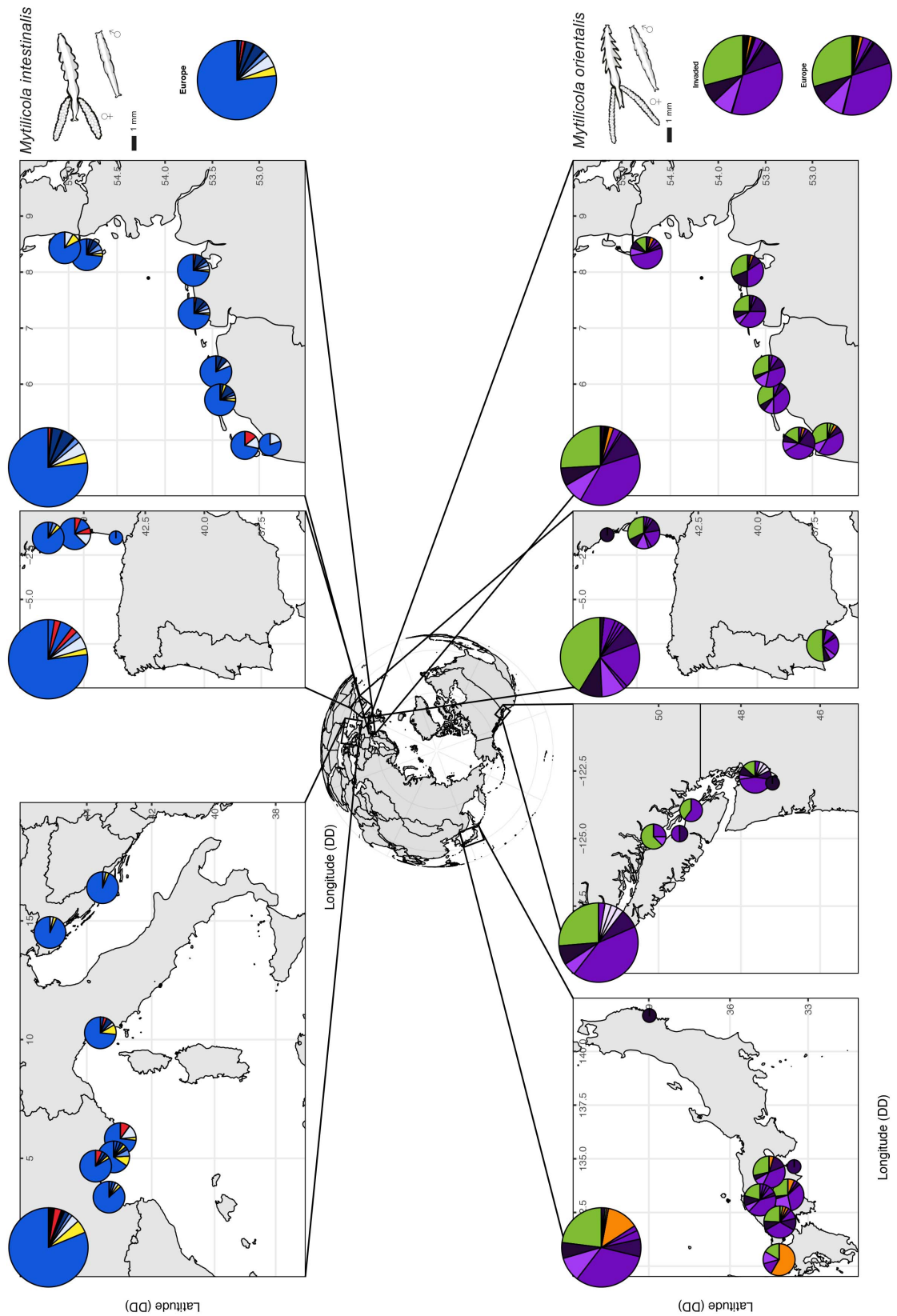
## Haplotype networks

The minimum spanning network of *M. intestinalis* has a star-like structure with one central, highly abundant haplotype from which several haplotypes are separated by few mutational steps (Figure 2.2). The star-like structure of the network suggests recent demographic expansion, as is also suggested by the negative Tajima's D and Fu's  $F_S$  values which were significant for most locations (Table 2.2) and by the mismatch distributions (Figure 2.4). Nine of the ten non-singleton haplotypes were shared between the putative native and invaded range (Figures 2.2 and 2.3, Table S2.1); only haplotype Mi16 was solely found in the invaded range (La Rochelle, Table S2.1).

In contrast, the minimum spanning network of *M. orientalis* was relatively complex, with two common frequent haplotypes that both showed associated derived haplotypes (Figure 2.2). With the exception of one location in Japan, all locations had one of the main haplotypes as its most frequent haplotype. A small group of related



**Figure 2.2** Minimum spanning networks for COI haplotypes (circles) in *Mytilicola intestinalis* and *M. orientalis*. Number of nucleotide differences is proportional to branch length (shortest branches represent one nucleotide difference) and black dots on the branches represent missing haplotypes. Frequency of observation is proportional to circle area. Colours represent different clusters of haplotypes and correspond with the pie charts in Figure 2.3.



**Figure 2.3** Maps showing pie charts of cytochrome-c-oxidase 1 (COI) haplotype frequencies in the invasive parasite species *Mytilicola intestinalis* and *Mytilicola orientalis*. Pie charts are smaller for samples with fewer than 15 individuals, which were not included in our analyses. Colours correspond to the different clusters of haplotypes in the minimum spanning networks presented in Figure 2.2.

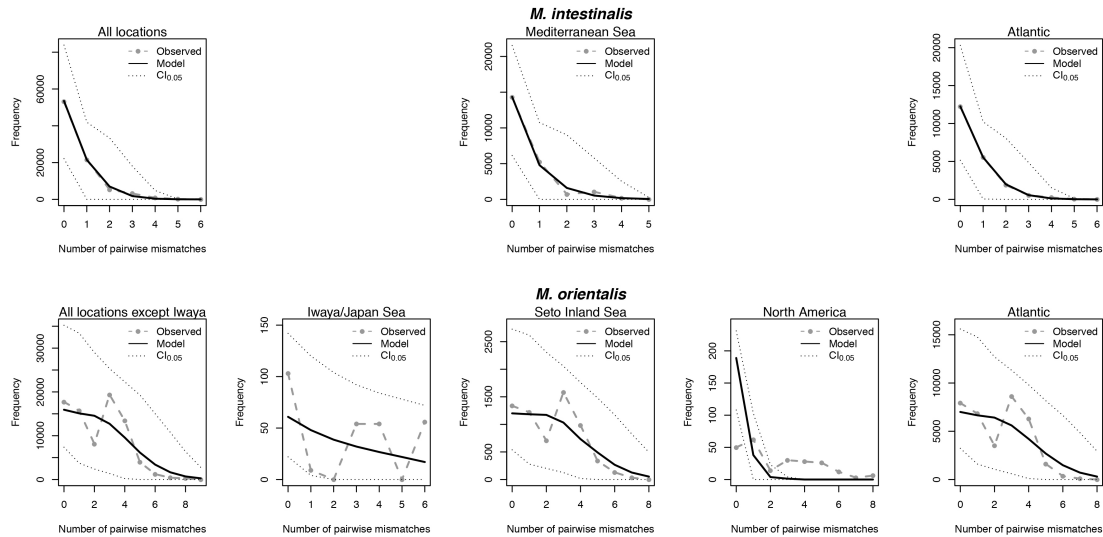
singleton haplotypes was found only in North America (light purple coloured haplotypes Mo11 – Mo13, Figures 2.2 and 2.3, Table S2.2). This type of network is more typically associated with stable demographics, also suggested by the non-significant Tajima's D and Fu's  $F_S$  values (Table 2.2) and by the mismatch distributions (Figure 2.4). Only one population (Puan Klent, Germany), representing the northern invasion front of *M. orientalis* stood out with significant negative Tajima's D and Fu's  $F_S$  values (Table 2.2).

### Population structure

For *M. intestinalis*, the overall level of population differentiation was estimated at  $\Phi_{ST} = 0.01468$  ( $p = 0.0108$ ) and  $F_{ST} = 0.00848$  (n.s.), with an overall population differentiation in the Mediterranean Sea at  $\Phi_{ST} = 0.01531$  ( $p = 0.0323$ ) and  $F_{ST} = 0.02397$  ( $p = 0.0325$ ), and in the invaded region at  $\Phi_{ST} = 0.01081$  (n.s.) and  $F_{ST} = -0.00544$  (n.s.), (Table 2.3, Table 2.4). The overall level of population differentiation for *M. orientalis* was higher and estimated at  $\Phi_{ST} = 0.05242$  ( $p < 0.00001$ ) and  $F_{ST} = 0.03002$  ( $p < 0.00001$ ), with an overall population differentiation in Japan at  $\Phi_{ST} = 0.10287$  ( $p < 0.00001$ ) and  $F_{ST} = 0.07019$  ( $p < 0.00001$ ), and in the introduced region at  $\Phi_{ST} = 0.02243$  ( $p = 0.0303$ ) and  $F_{ST} = 0.01005$  (n.s.) (Table 2.3, Table 2.4).

The comparatively small overall genetic differentiation in *M. intestinalis* could not be attributed to any single location or group of locations based on the pairwise population comparisons, because none of the latter were significant after Bonferroni correction (Table S2.3, Table S2.4). The hierarchical AMOVAs did not clarify this. Our analyses indicated that Cassis should be grouped with the Ligurian Sea (negative  $\Phi_{CT}$  in Table 2.5). With Cassis grouped in the Ligurian Sea, the percentage of variation among groups was 0.38%, among populations within groups 1.24% and within populations 98.38%, resulting in  $\Phi_{CT} = 0.0038$  (n.s.),  $\Phi_{SC} = 0.01247$  (n.s.) and  $\Phi_{ST} = 0.0162$  ( $p = 0.0480$ ) (Table 2.5). Using classical F-statistics, the percentage of variation among groups was greater at 2.07%, among populations within groups smaller at 0.84% and within populations 97.12%, resulting in  $F_{CT} = 0.02069$  (n.s.),  $F_{SC} = 0.00825$  (n.s.) and  $F_{ST} = 0.02878$  ( $p = 0.317$ ) (Table 2.6). In both hierarchical AMOVAs using mitochondrial statistic  $\Phi$  and conventional F-statistic, there was no significant difference between the Gulf of Lion, the Ligurian Sea and the Adriatic Sea. Therefore, the small amount of population structure that was detected in the





**Figure 2.4** Mismatch distributions of *Mytilicola intestinalis* (top row) and *Mytilicola orientalis* (lower row). From left to right: mismatch distributions based on population differences (*M. intestinalis*: all locations pooled; *M. orientalis*: Iwaya separate from the rest of the locations), mismatch distributions in the putative native region and in the invaded region(s). The sharp decline in *M. intestinalis* is indicative of population expansion after a bottleneck or selective sweep; the bimodal distributions in *M. orientalis* are indicative of stable demographics.

**Table 2.2** Tajima's D and Fu's  $F_s$  neutrality tests for partial cytochrome-*c*-oxidase 1 sequences in *Mytilicola intestinalis* and *M. orientalis*, based on 10,000 simulations. Level of significance is indicated ( $p < 0.05$  \*,  $p < 0.01$  \*\* and  $p < 0.001$  \*\*\*). Only samples with more than 15 individuals were included in the analyses.

Locations	Tajima's D	Fu's $F_s$	Locations	Tajima's D	Fu's $F_s$
<b><i>M. intestinalis</i>, putative native region</b>			<b><i>M. orientalis</i>, putative native region</b>		
Marjan, Croatia	-1.509 *	-2.312 **	Iwaya, Japan	1.24	3.06
Malinska, Croatia	-1.507 *	-2.355 *	Ondo, Japan	-0.482	-1.11
Livorno, Italy	-1.871 *	-3.027 **	Takehara, Japan	-1.12	-2.23
Cassis, France	-0.8597	0.0501	Niihama, Japan	-0.372	-0.109
Istres, France	-1.749 **	-2.054	Kagawa, Japan	-0.220	-0.0780
Rouet, France	-1.662 *	-5.145 ***			
Bouzigues, France	-1.889 **	-4.411 ***			
<b><i>M. intestinalis</i>, invaded region</b>			<b><i>M. orientalis</i>, invaded region</b>		
La Rochelle, France	-0.9778	-0.7836	Belfair, USA	-1.12	-2.22
La Tremblade, France	-1.878 **	-2.206 *	Tavira, Portugal	0.125	0.110
Ameland, Netherlands	-1.658 *	-4.526 **	Arcachon, France	-0.878	-1.15
Schiermonnikoog, Netherlands	-1.349	-1.867 *	Balgzand, Netherlands	-0.230	-0.319
Norddeich, Germany	-1.638 *	-3.161 *	De Cocksdorp, Netherlands	-1.04	-1.50
Horumersiel, Germany	-1.159	-1.259 *	Ameland, Netherlands	0.0919	0.518
Puan Klent, Germany	-1.758 *	-4.899 ***	Schiermonnikoog, Netherlands	-0.208	-0.266
Königshafen, Germany	-0.864	-0.8722	Norddeich, Germany	-0.420	-0.558
			Horumersiel, Germany	0.178	0.655
			Puan Klent, Germany	-1.538 *	-3.22 *

**Table 2.3** Results from analyses of molecular variance (AMOVA) on partial cytochrome-*c*-oxidase 1 (COI) sequences in *Mytilicola intestinalis* and *M. orientalis*. Only samples with more than 15 individuals were included. d.f. degrees of freedom; SS sum of squares; VC variance component,  $\Phi_{ST}$  overall fixation index.

Locations		d.f.	SS	VC	$\Phi_{ST}$	<i>p</i> -value
<b><i>M. intestinalis</i></b>						
All	among	14	5.318	0.00402		
	within	396	106.955	0.27009		
	total	410	112.273	0.27411	0.01468	0.01075
Mediterranean	among	6	2.147	0.00381		
	within	201	49.199	0.24477		
	total	207	51.346	0.24858	0.01531	0.03226
Introduced	among	7	2.638	0.00324		
	within	195	57.756	0.29618		
	total	202	60.394	0.29942	0.01081	0.10264
<b><i>M. orientalis</i></b>						
All	among	14	39.374	0.06064		
	within	410	449.440	1.09620		
	total	424	488.814	1.15683	0.05242	<0.00001
Japan	among	4	18.935	0.13168		
	within	132	151.576	1.14830		
	total	136	170.511	1.27998	0.10287	<0.00001
Introduced	among	9	16.007	0.02458		
	within	278	297.865	1.07146		
	total	287	313.872	1.09604	0.02243	0.03030

**Table 2.4** Results from analyses on molecular variance (AMOVA) on partial cytochrome-*c*-oxidase 1 (COI) sequences in *Mytilicola intestinalis* and *M. orientalis* with conventional F-statistics. Only samples with more than 15 individuals were included. d.f. degrees of freedom; SS sum of squares; VC variance component,  $F_{ST}$  overall fixation index.

Locations		d.f.	SS	VC	$F_{ST}$	<i>p</i> -value
<b><i>M. intestinalis</i></b>						
All	among	14	3.167	0.00157		
	within	396	72.622	0.18339		
	total	410	75.622	0.18496	0.00848	0.13436
Mediterranean	among	6	1.707	0.00404		
	within	201	33.062	0.16449		
	total	207	34.062	0.16853	0.02397	0.03248
Introduced	among	7	1.228	-0.00110		
	within	195	39.560	0.20287		
	total	202	40.788	0.20177	-0.00544	0.63683
<b><i>M. orientalis</i></b>						
All	among	14	10.084	0.01188		
	within	410	157.436	0.38399		
	total	424	167.520	0.39587	0.03002	<0.00001
Japan	among	4	4.672	0.02866		
	within	132	50.459	0.38227		
	total	136	55.131	0.41112	0.07019	<0.00001
Introduced	among	9	4.475	0.00391		
	within	278	106.977	0.38481		
	total	287	111.451	0.38871	0.01005	0.11604

overall test cannot clearly be attributed to one of the Mediterranean basins, nor to one or a few individual locations.

In contrast to *M. intestinalis*, there was strong and significant genetic differentiation between locations in the native region of *M. orientalis*. Iwaya, located in the Sea of Japan, was strongly differentiated from nearly all other sampling locations along the Seto Inland Sea ( $\Phi_{ST} = 0.215 - 0.290$ , Table S2.5;  $F_{ST} = 0.165 - 0.216$ , Figure S2.3, Table S2.6). Only the pairwise population comparison between Iwaya and Kagawa was not significant after Bonferroni correction ( $\Phi_{ST} = 0.220$ ,  $p =$

**Table 2.5** Results from hierarchical analyses of molecular variance (AMOVA) based on pairwise differences, on partial cytochrome-*c*-oxidase 1 (COI) sequences in Mediterranean *Mytilicola intestinalis*. Only samples with more than 15 individuals were included. d.f. degrees of freedom; SS sum of squares; VC variance component, % var percentage of variation.

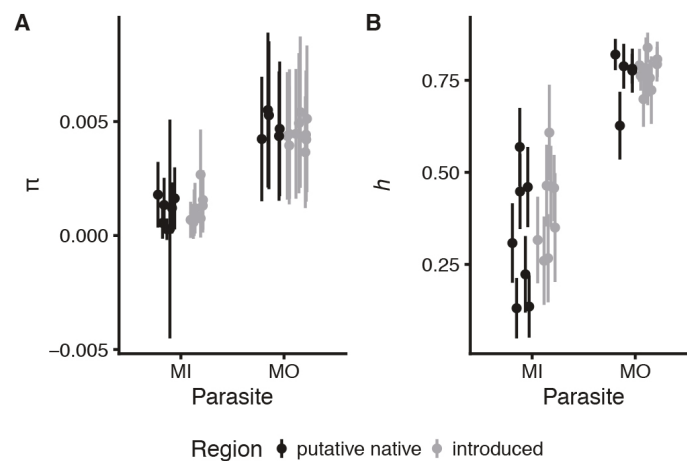
	d.f.	SS	VC	% var	<i>p</i> -value	$\Phi$ -statistic
<b>AMOVA 1: Gulf of Lion (Bouzigues, Istres, Rouet, Cassis) vs. Ligurian Sea (Livorno) vs. Adriatic Sea (Marjan, Malinska)</b>						
among groups	2	0.476	-0.00303	-1.22	0.76653	$\Phi_{CT} = -0.01224$
among populations						
within groups	4	1.671	0.00583	2.35	0.04089	$\Phi_{SC} = 0.02325$
within populations	201	49.199	0.24477	98.87	0.04465	$\Phi_{ST} = 0.01130$
total	207	51.346	0.24757			
<b>AMOVA 2: Gulf of Lion (Bouzigues, Istres, Rouet) vs. Ligurian Sea (Cassis, Livorno) vs. Adriatic Sea (Marjan, Malinska)</b>						
among groups	2	0.801	0.00094	0.38	0.33257	$\Phi_{CT} = 0.00378$
among populations						
within groups	4	1.346	0.00309	1.24	0.14168	$\Phi_{SC} = 0.01247$
within populations	201	49.199	0.24477	98.38	0.04802	$\Phi_{ST} = 0.011620$
total	207	51.346	0.24880			

**Table 2.6** Results from hierarchical analyses of molecular variance (AMOVA) on partial cytochrome-*c*-oxidase 1 (COI) sequences in Mediterranean *Mytilicola intestinalis* with conventional *F*-statistics. Only samples with more than 15 individuals were included. d.f. degrees of freedom; SS sum of squares; VC variance component, % var percentage of variation.

	d.f.	SS	VC	% var	<i>p</i> -value	<i>F</i> -statistic
<b>AMOVA 1: Gulf of Lion (Bouzigues, Istres, Rouet, Cassis) vs. Ligurian Sea (Livorno) vs. Adriatic Sea (Marjan, Malinska)</b>						
among groups	2	0.776	0.00261	1.54	0.20634	$F_{CT} = 0.01543$
among populations						
within groups	4	0.931	0.00230	1.36	0.13525	$F_{SC} = 0.01378$
within populations	201	33.062	0.16449	97.10	0.03040	$F_{ST} = 0.02899$
total	207	34.769	0.16940			
<b>AMOVA 2: Gulf of Lion (Bouzigues, Istres, Rouet) vs. Ligurian Sea (Cassis, Livorno) vs. Adriatic Sea (Marjan, Malinska)</b>						
among groups	2	0.887	0.00350	2.07	0.16406	$F_{CT} = 0.02069$
among populations						
within groups	4	0.821	0.00137	0.84	0.23287	$F_{SC} = 0.00825$
within populations	201	33.062	0.16449	97.12	0.03168	$F_{ST} = 0.02878$
total	207	34.769	0.16936			

0.00129 > Bonferroni corrected  $p$ -level of 0.000476, Table S2.5), but with conventional F-statistics also this pairwise comparison was significantly different ( $F_{ST} = 0.191$ ,  $p = 0.00040 < \text{Bonferroni corrected } p\text{-level of } 0.000476$ , Table S2.6). No genetic differentiation was detected between the locations sampled along the Seto Inland Sea, i.e., Takahara, Ondo, Niihama and Kagawa. All pairwise population comparisons between Sea of Japan (Iwaya) and locations from the invaded regions in North America and Europe were significant after Bonferroni correction and showed strong genetic differentiation ( $\Phi_{ST} = 0.228 - 0.344$ , Table S2.5;  $F_{ST} = 0.187 - 0.247$ , Table S2.6). No significant pairwise population differences were detected between locations in the invaded regions.

We did not detect significant differences in haplotype diversity ( $h$ ) between putative native and invaded regions for *M. intestinalis* (ANOVA,  $df = 1$ ,  $F = 1.1082$ ,  $p = 0.302$ ) (Figure 2.5), with  $h$  ranging from 0.131 to 0.569 in putative native region and from 0.260 to 0.608 in the invaded region for *M. intestinalis* (Table S2.1) and  $h$  ranging from 0.627 to 0.820 in Japan and from 0.699 to 0.839 in the introduced region for *M. orientalis* (Table S2.2). Similarly, no significant differences were detected for nucleotide diversity ( $\pi$ ) between the putative native region and the invaded regions of *M. intestinalis* ( $p = 0.552$ ), or *M. orientalis* (ANOVA,  $F = 0.0284$ ,  $d.f. = 1$ ,  $p = 0.867$ ). Nucleotide diversity for *M. intestinalis* ranged from 0.000276 to 0.00179 in the putative native region, and from 0.000572 to 0.00267 in the introduced region (Figure 2.5, Table S2.1), and *M. orientalis*' nucleotide diversity ranged from 0.00423



**Figure 2.5** Nucleotide diversity  $\pi$  and haplotype diversity  $h$  of *Mytilicola intestinalis* (MI) and *M. orientalis* (MO) in putative native region (grey) and invaded region (black). Bars represent standard deviation. Only samples with more than 15 individuals were included. See Tables S2.1 and S2.2 for  $\pi$  and  $h$  values of individual locations underlying this figure.

to 0.00550 in Japan and from 0.00366 to 0.00541 in the introduced regions (Figure 2.5, Table S2.2). However, both haplotype diversity and nucleotide diversity were significantly larger for *M. orientalis* than for *M. intestinalis* (ANOVA  $h$ :  $F = 99.5662$ , d.f. = 1,  $p < 0.00001$ ; ANOVA  $\pi$ :  $df = 1$ ,  $F = 251.7502$ ,  $p < 0.00001$ ). The interaction term in the linear models (parasite species  $\times$  region) was not significant for  $h$  or  $\pi$  (ANOVA  $h$ :  $F = 0.5634$ , d.f. = 1,  $p = 0.460$ ; ANOVA  $\pi$ :  $F = 1.3178$ , d.f. = 1,  $p = 0.261$ ) and optimizing the models with the step function based on AIC would have removed the interaction term and region term from both models, indicating that they were not predictive of differences in genetic diversity.

## Discussion

Our study utilises the consecutive invasions of two congeneric invasive parasites to align the invasion history derived from their reported spread in the literature with their phylogeography. It shows that despite a large body of literature existing for these conspicuous and common marine parasites of commercially important bivalves, hypotheses derived from such reports do not necessarily match conclusions based on population genetic demography. This indicates that some basic understanding of the invasion processes may lack and highlights the use of population genetic studies to test the seemingly obvious. We show that native region and invasion route of *Mytilicola orientalis* matches the classical pattern of a recent invasion described in the literature, while the phylogeography of *Mytilicola intestinalis* did not match our literature based expectations, probably making it a cryptogenic species (i.e., a species whose origin cannot readily be determined with the available data, *sensu* Carlton 1996) in the Mediterranean Sea.

### Literature survey

*Mytilicola intestinalis* and *M. orientalis* had different modes of invasion. The invasion of *M. intestinalis* can be characterised by local introductions at seaports and harbour areas and slow natural spread, whereas the invasion of *M. orientalis* was extremely long-distance via aquaculture transports, followed by a relatively rapid spread in Europe due to these transports, and its local invasion is still ongoing (*Chapter 3*). The wide host range of *M. orientalis* and spillover to native bivalves (Goedknecht *et al.*

2017a) could further speed up its spread compared to that of *M. intestinalis*. Interestingly, the distribution of *M. orientalis* in the Mediterranean is disjunct, with presence at the Thau lagoon since 1979 (Clanzig 1989), but absence east of this location along the French and Italian coasts (Table 2.1), and again a presence in the northern Adriatic (Kovačić *et al.* 2016). This probably reflects the long-distance transports of oysters and the ongoing invasion of *M. orientalis*, while it also shows that *Mytilicola* spp. do not colonise over large distances unaided by anthropogenic factors.

A caveat in the literature survey is that *M. orientalis*' invasion in Europe has been cryptic because of difficulties with reliable species identification (Goedknecht *et al.* 2018). In the literature survey, we went with the identification presented in each of the papers, but that may be incorrect when studies are based on histology, as the distinguishing characteristics for *M. intestinalis* and *M. orientalis* are not fully reliable (Goedknecht *et al.* 2018). For instance, *M. intestinalis* has been reported from *Magallana gigas* (Aguirre-Macedo & Kennedy 1999; Rementeria *et al.* 2017), although it was shown not to be a suitable host for *M. intestinalis* (Dare 1982, Chapter 3); these observations are more likely to be of *M. orientalis* instead.

### **Native region and invasion route of *Mytilicola orientalis***

Our study confirmed Japan as the native region of *M. orientalis*, because of the significant population genetic structure between the Seto Inland Sea and Iwaya in the Sea of Japan, and the stronger overall population differentiation in Japan than in the invaded region. This was expected based on our literature survey. Pacific oysters have been imported from Japan across the globe, with Miyagi as the main overseas export area and with Kumamoto and Hiroshima contributing smaller amounts (Ventilla 1984), and *M. orientalis* was reported as their parasitic hitchhiker in North America (Odlag 1946) and in Europe (His 1977a, b; His *et al.* 1978). Oyster culture practices in Japan date back to the beginning of the seventeenth century in the Hiroshima Bay area at the Seto Inland Sea (Ventilla 1984), and this is still the main producer of oysters in Japan at two thirds of the total production (Kawamura *et al.* 2017). Oyster seed has been distributed from these aquaculture areas to others within the country (Ventilla 1984). *Magallana gigas* in the main aquaculture areas (Hiroshima, Okayama, Miyagi) are genetically close despite their geographic distance, while *M. gigas* has regionally restricted gene flow in Nagasaki and the Kumano regions

(Kawamura *et al.* 2017). Results from the population genetic analyses presented here are consistent with these patterns in the native range and invaded regions: the North American and European samples could not be distinguished from the Seto Inland Sea samples from Japan, while the sample from Iwaya in the Sea of Japan was significantly different.

Because Pacific oysters have been imported from Miyagi, it logically follows that *M. orientalis* should have been present there. As far as we know, there are no records about *M. orientalis* presence from this area. The first specimens in north Japan were found during the current study in Ofunato, Sakihama and Onizawa (all in the Iwate prefecture), however, because the prevalences were so low, we unfortunately could not obtain a full sample to include in the invasion genetics analyses. Anecdotally, the single sequenced individual from Ofunato did not belong to the two main haplotypes found in the study, but belonged to the fifth most frequent haplotype (haplotype Mo2). This region was affected by the Great East Japan Earthquake in 2011, which caused serious damage to the oyster aquaculture along the Pacific coast of North Japan (Kawamura *et al.* 2017 and references therein).

Pacific oysters form two distinct groups in Europe, with northern populations in Germany, Denmark and Sweden, originating from British Columbia imports, showing less diversity and a stronger genetic structure, while southern populations, originating from Japanese imports, show no such decrease in genetic diversity (Rohfritsch *et al.* 2013). Interestingly, contrary to *M. gigas*' invasion in the Wadden Sea where samples from North America resembled samples from the southern Wadden Sea invasion (Moehler *et al.* 2011), *M. orientalis* showed more resemblance in the MDS plots between North America and the northern Wadden Sea invasion: Belfair (representing North America) and Puan Klent (representing the northern Wadden Sea oyster invasion). This may suggest that secondary spread of *M. orientalis* by other vectors than oyster transport has also contributed to its invasion within the Wadden Sea. However, none of these pairwise population comparisons were significant, meaning that this cannot be said with any certainty. From our literature survey it became clear that by the time *M. orientalis* was discovered in the Wadden Sea, it was already present across a large area, whereas the invasion of *M. intestinalis* throughout the Wadden Sea took decades. Likely this difference can be dedicated to the co-introduction of *M. orientalis* with Pacific oysters, which took only a few years to invade the Wadden Sea (Troost 2010), while *M. intestinalis* presumably invaded by

natural dispersal. Whether *M. orientalis* was introduced to Europe via Canada, or directly from Japan, or both, and how *M. orientalis* spread throughout Europe remains to be answered with the higher resolution of more independent genetic markers. *Mytilicola orientalis* was likely co-introduced in large numbers or via multiple introductions, because of the equally high genetic diversity in native and invaded regions. Thus, the invasion history from the native region along the reported invasion route of *M. orientalis* from our literature survey matches well with the resulting phylogeography.

### **Native region and invasion route of *Mytilicola intestinalis***

While *Mytilicola intestinalis* was always thought to originate from the Adriatic and Mediterranean coasts, our genetic data could not confirm from which location(s) *M. intestinalis* invaded the Atlantic coasts of Europe. None of the pairwise population comparisons were statistically significant after Bonferroni corrections. Even though the overall level of population genetic structure in the Mediterranean was significant, it was very low, probably owing to the low overall genetic diversity. Zooming in on this small difference, however, we did not detect a significant difference among Mediterranean basins based on hierarchical AMOVAs. The absence of clear population genetic differentiation in the Mediterranean is striking, for two reasons. First and foremost, if the Mediterranean Sea were the native distribution range of *M. intestinalis*, the long geographical history of the basins should have resulted in significant population structure in the present day. Second, the limited potential for natural dispersal of *M. intestinalis* by means of its short-lived pelagic larvae (Bolster 1954; Korryng 1968) should have contributed to maintaining population differentiation among the long-established Mediterranean populations. Although no uniform phylogeographic pattern in other marine taxa in the Mediterranean Sea was found (Patarnello *et al.* 2007), for many species the topography and oceanographic conditions of the Strait of Otranto and the Siculo-Tunisian Strait represent a break that typically causes population genetic differentiation between the Adriatic, Ionian and Ligurian Seas (e.g., Borrero-Pérez *et al.* 2011; Ladoukakis *et al.* 2002; Penant *et al.* 2013). Likewise, genetic breaks were found near Marseilles, separating the Ligurian Sea and Gulf of Lion especially for species with lower dispersal ability (Boissin *et al.* 2015; Cahill *et al.* 2017; Penant *et al.* 2013). Furthermore, breaks in the distribution of mussel hosts are known to be a barrier for *M. intestinalis* larval dispersal, likely



because of the short pelagic larval phase of the parasite (Korringa 1968). There are several possible explanations for the observed absence of population genetic differentiation between the Mediterranean *M. intestinalis* samples and the overall low genetic diversity compared to *M. orientalis*, which are detailed below.

#### *Natural dispersal*

First, the natural dispersive ability of *M. intestinalis* could be much greater than previously thought, with locations across the different Mediterranean basins closely connected. The nauplius and metanauplius stages are positively phototropic (Caspers 1939; Meyer & Mann 1951; Pesta 1907; Steuer 1905) and last 36 to 48 hours at a laboratory temperature of 18 °C (Bolster 1954). The next life stage, the infective copepodite stage, is not or negatively phototropic and may live for 11 to 15 days under laboratory circumstances, although it is doubtful whether it lives that long in the field (Bolster 1954). Once the *Mytilicola* has infected a host, its dispersive capacity depends on its bivalve host, which is sessile and does not move across long distances unaided. Therefore, the natural dispersive ability of *M. intestinalis* has been thought of as very limited (Bolster 1954; Korringa 1968). This is further illustrated by the invasion data of *M. intestinalis*, which show a slow spread upon invasion in, for instance, the Dutch Wadden Sea and Scottish lochs. The slow advance of the invasion front does not necessarily dictate that gene flow at the scale of Mediterranean basins must be low; it must be noted that gene flow among established populations may be higher than into new territory, because of factors including finding suitable mates and susceptible hosts. In that case, an isolation-by-distance scenario may be expected, but this is not reflected in the data because of the lack of any structure. Finally, population differentiation was found for congener *M. orientalis*, which has a very similar life cycle and may therefore be expected to have similar dispersal capacities. Natural dispersal, therefore, is likely not the main explanation for the lack of population genetic structure in *M. intestinalis* in the Mediterranean Sea.

### *Selective sweep and demographic processes*

Second, a bottleneck or selective sweep in *M. intestinalis* may have occurred prior to its invasion, which reduced variation or in which a mitochondrial mutant had a selective advantage and spread throughout the native range, erasing any existing population differentiation. Such a scenario is supported by the starlike haplotype network, relatively low genetic diversity and significant negative Tajima's D statistic that indicates recent population expansion or recovery from an ancient selective sweep, after which diversity slowly recovered, creating a starlike haplotype network at the start. Low gene flow between locations throughout the Mediterranean, via natural dispersal or via anthropogenic displacement, should theoretically be less of a barrier towards the spread of an advantageous mutation than to the building up of neutral population structure. Here, studying additional independent (nuclear DNA) markers will help to disentangle possible selective events from demographic processes.

### *Anthropogenic dispersal*

Third, anthropogenic shuffling of native genetic diversity might explain the lack of population genetic structure in *M. intestinalis* within the Mediterranean. Mankind could have aided its dispersal, for instance, by transporting infected hosts among aquaculture sites, which was the vector of introduction for *M. orientalis*. However, the typically small size of translocated mussels ('spat') contests this. The number of parasites is usually proportional to the shell length of mussels (Andreu 1960; Grainger 1951) and very young mussels are rarely infected (Korringa 1968). Translocated mussel spat under 20 mm long may harbor *M. intestinalis*; in a heavily infested *M. edulis* bed, the smallest infected mussel was 9.9 mm long and young mussels >12 mm were more commonly infested (Williams 1967). The relaying of infected mussels from the German Wadden Sea is how *M. intestinalis* invaded the mussel beds and aquaculture in Zeeland, the Netherlands (Korringa 1950). However, Mediterranean commercial mussels are usually grown in hanging cultures, which are considerably less vulnerable for parasite infections than mussels grown on the bottom. They are also replaced yearly by a new parent stock, which makes it difficult for *M. intestinalis* to build up a dense population (reviewed in Korringa 1968). Nevertheless, transportation of spat between Mediterranean regions does occur and could thus be

contributing or have contributed in the past to the homogenization of its parasite fauna.

Other human vectors of mussel dispersal are ballast water and ship hull fouling (Wonham 2004), the latter being seen as the primary vector by which *M. intestinalis* invaded the German and British coasts in the first place (e.g., Bolster 1954) and explaining the disjunct distribution on *M. intestinalis* there (reviewed in Korrynga 1968). While anthropogenic shuffling could explain the lack of population genetic structure in the Mediterranean and Adriatic Seas, it would not explain the overall low genetic diversity compared to *M. orientalis*. Moreover, a genetically differentiated population of host species *Mytilus galloprovincialis* was found at the Croatian coast (Giantsis *et al.* 2014), adding further doubt to anthropogenic shuffling as the main cause of the lack of population genetic structure in Mediterranean *M. intestinalis*.

#### *Native origin is not the Mediterranean*

Fourth and finally, *M. intestinalis* may in fact be an introduced species in the Mediterranean Sea in the range it is currently known for, which would explain the lack of population genetic structure and the indications for population expansion. Also, a population bottleneck upon invasion could explain the lack of genetic variation. A few hints can be found in literature for this scenario.

*Mytilicola intestinalis* was described in 1902 and 1903 from two important ports for shipping, Trieste and Gravosa (both Adriatic Sea) (Steuer 1902, 1905). This is around 40 years after the opening of the Suez Canal (November 1869), which led to the invasion of many marine species into the Mediterranean, an invasion process commonly referred to as the Lessepsian migration (e.g., Galil 2000; Galil *et al.* 2014). In 1902 nearly all dissected mussels at those Adriatic sites were infected by *M. intestinalis*, with up to 50 parasites in one host (Steuer 1902, 1905). Prevalences and intensities that high are typically seen in mussel populations that were recently invaded – e.g., maximum intensity of up to 14 parasites in one host in Germany in 1938 (Caspers 1939), up to 59 in the UK (Cole & Savage 1951) and up to 70 in the Netherlands (Korrynga 1951). In 1953 *M. intestinalis* could not be found in the same locations where A. Steuer initially described the species (Meyer-Waarden & Mann 1953). In our own dissections from Trieste we were also not able to find *M. intestinalis* in >200 mussels. Furthermore, Cerruti (1932) notes that a few years prior to their publication, *M. intestinalis* was a sudden pest in Mare Piccolo, Taranto (Italy)

and that, by undertaking actions rapidly and effectively, authorities were able to successfully eradicate the parasite. In 1953, Meyer-Waarden and Mann (1953) found a prevalence of 6.7% and a mean infection intensity of 1.8 in 106 dissected mussels from the same site, and in 1996 *M. intestinalis* was absent from 31 dissected mussels (Canestri Trotti *et al.* 1998). The cases of Trieste and Taranto both may be interpreted as outbreaks upon initial invasion followed by low infestation at the longer time scale, resembling the typical boom-bust dynamics of biological invasions before populations enter the comparatively stable adjustment phase (Reise *et al.* 2006).

Moreover, Hrs-Brenko (1964) noted that the areas where large ships rarely stop in the Adriatic Sea (Mali Ston, Klimno, Novigrad Sea) show no or low *M. intestinalis* prevalence, while mussels at large ports were all heavily infested (Koper, Sibenik, Izola, Reijiki, Zadar, Cres). Also, Giusti (1967) observed that prevalence of *M. intestinalis* decreased with distance from shipping ports at Livorno and Carrara (Italy), both of which have abundant commercial activities, and concluded that the distribution of *M. intestinalis* can be explained by level of wave exposure, distance to blue mussel farms and distance to shipping ports. In Greece, the distribution of *M. intestinalis* is disjunct, being present in cultured mussels in the Thermaikos Gulf (Karagiannis *et al.* 2013; Rayyan *et al.* 2004), but absent in all of our Greek samples from natural areas. These observations are consistent with a scenario in which *M. intestinalis* is not native in the Mediterranean, but has invaded with ship hull fouling and/or aquaculture transports as vector. On the other hand, these observations are also consistent with recent colonization from an unknown Mediterranean refugium and ship transport, where high-density situations in farms near ports would be beneficial for transmission.

Lastly, two female *M. intestinalis* were found as free-living specimens in a plankton sample taken in the Indian Ocean-Malacca Strait area (Wickstead 1960). The occurrence of this parasite at open sea is highly unusual and could indicate that these individuals came from ship fouling (Wickstead 1960), highlighting the potential of this invasion vector. Interestingly, a *Mytilicola* spp. was found in *Crassostrea madrasensis* oysters on the south-west coast of India (Suja *et al.* 2017), but because histopathological methods were used, the authors were not able to determine the sample to species level (Suja, pers. comm.). We are not aware of any other records of a *Mytilicola* species from this part of the world. These two observations may be

indicative of a native region of *M. intestinalis* somewhere in the Indian Ocean, but clearly this is highly speculative without further research.

Ships may have carried infected hosts on hulls through the Suez Canal between time of opening and *M. intestinalis* species description, akin to its invasion into the eastern North Atlantic. However, *M. galloprovincialis* does not occur in the far eastern Mediterranean or the Red Sea, so when this scenario applies, *M. intestinalis*' original host must have been a different one. *Modiolus barbatus* Linnaeus, 1758 stands out as a potential native host for *M. intestinalis*, because this species' range extends further into the eastern Mediterranean than *M. galloprovincialis* (Barash & Danin 1982) and because *M. intestinalis* has been found in this species before (Bocquet & Stock 1957; Mladineo 2008). The higher salinity in the eastern Mediterranean and Red Sea should be no problem for *M. intestinalis*, neither should the higher temperatures, as the parasite is tolerant to a wide range of environmental conditions (reviewed in Korryng 1968). We are aware of only two publications describing the absence/presence of *M. intestinalis* on the southern coasts of the Mediterranean, both of which were conducted in the Bizerta Bay, Tunisia. There, *M. intestinalis* was absent in imported French *M. edulis* (Heldt 1951) and also absent in the date mussel *Lithophaga lithophaga* Linné, 1758 (Jaafar Kefi *et al.* 2012). Furthermore, on the island of Rhodes in the eastern Mediterranean, we did not detect *M. intestinalis* in 90 *Brachodontes pharoensis*. As far as we know, there have been no studies on the potential presence or absence of *M. intestinalis* in the Red Sea.

#### *Cryptogenic species*

The data presented here did not confirm that the Mediterranean Sea is the native region of *M. intestinalis*. The results can be explained by demographic processes, by a selective sweep, by natural or human-aided dispersal and by *M. intestinalis* being an invader in its currently known range in the Mediterranean. None of these explanations are mutually exclusive and they each require further investigation. Because our data may not be consistent with the idea that *M. intestinalis* is native in the Mediterranean Sea, we argue that *M. intestinalis* should be regarded as a cryptogenic species (i.e., a species whose origin cannot readily be determined with the available data, *sensu* Carlton 1996). Along the Atlantic and North Sea coasts of Europe, however, *M. intestinalis* is clearly an invasive species, as there are records of times when *M. intestinalis* was not present, and later invaded. The absence of such data in the

Mediterranean neither proves nor disproves presence or absence of *M. intestinalis* in the Mediterranean before its description. The higher prevalence around shipping ports and aquaculture areas could indicate that *M. intestinalis* is invasive, or that its distribution has been influenced by humans on the long-term. Long-distance transport of Pacific oysters led to the introduction of *M. orientalis*, highlighting this vector's potential for parasite invasion. Solving the puzzle whether *M. intestinalis* is native or is an invader in the Mediterranean would need explorative, basic parasitological work in potential native regions such as the southern and eastern Mediterranean Sea, the Red Sea and potentially further, in combination with a population genetic study with the genetic marker currently used and, preferably, additional independent (nuclear) markers.

## Conclusion

Our study shows that even though these two invasive parasites have been studied relatively intensively, basic knowledge can still be lacking. The native region and invasion route of *M. orientalis* were validated with the sampling and choice of marker in the current study: the native region of *M. orientalis* is Japan, and the parasite hitchhiked with its host to North America and Europe. Within Japan, culturing areas in or closely connected to those in the Seto Inland Sea are the likely source of invasion. In contrast, the native region of *M. intestinalis* could not be validated, because of low genetic diversity and lacking population structure within and among the Mediterranean basins. While different explanations for this pattern can be given, i.e., high connectivity within the Mediterranean, or a selective sweep, we cannot rule out the possibility that *M. intestinalis* is in fact an invader in the Mediterranean. Hence, *M. intestinalis* should be regarded as a cryptogenic species in the Mediterranean and as an invasive species on the eastern North Atlantic coasts. Should *M. intestinalis* be an invader in the Mediterranean, its native host species is likely not *M. galloprovincialis*. We could confirm the suspicions that *M. orientalis* co-invaded in large numbers, and that *M. intestinalis* invaded more in its own. The invasion histories and demographics thus differ between the two *Mytilicola* species, and this may have profound impacts on their further invasions and evolutionary trajectories, as well as their competition in the invaded regions where they now co-occur.

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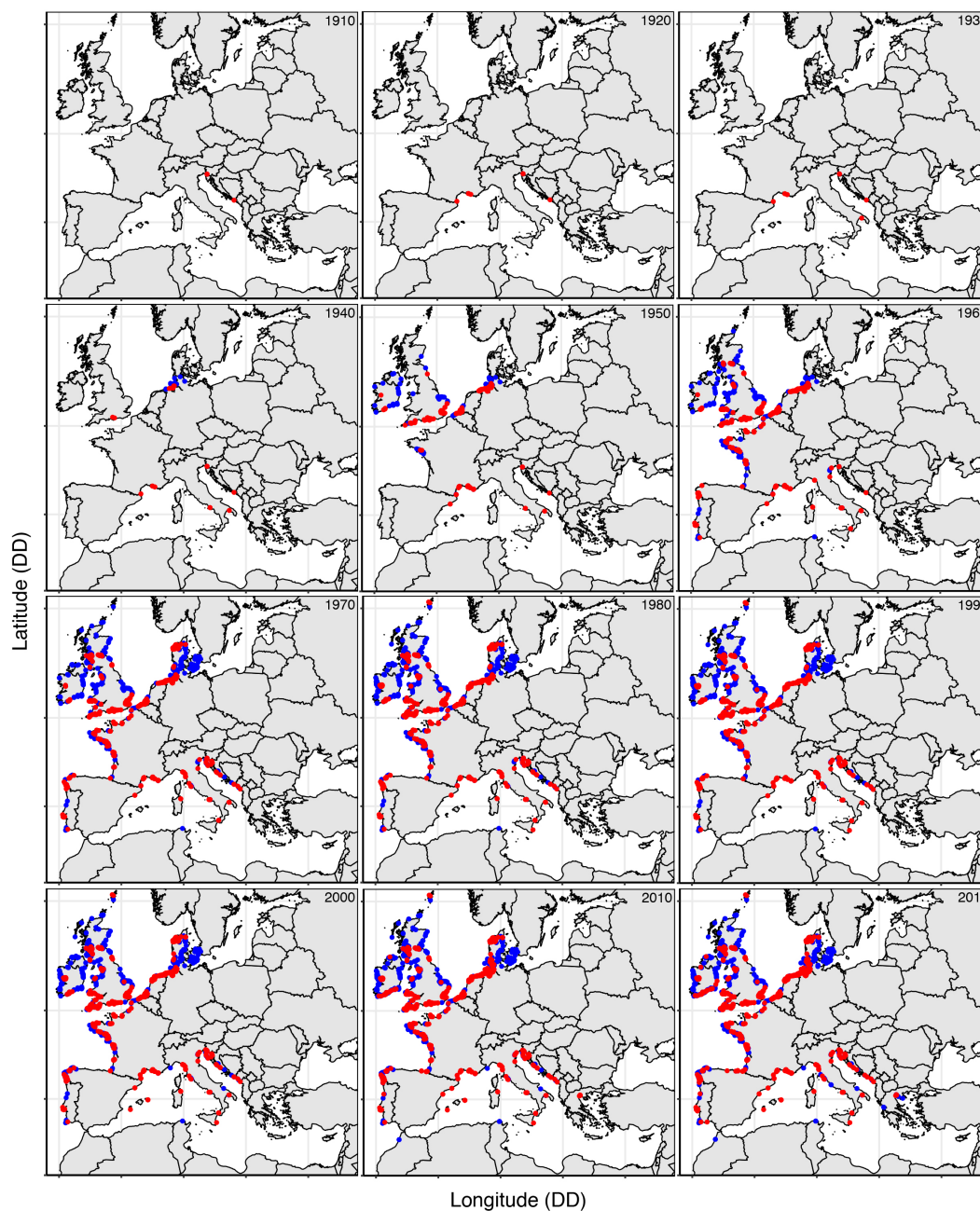
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## Supplementary materials

### *Mytilicola intestinalis*

Presence

- Absent
- Present



**Figure S2.1** Cumulative *Mytilicola intestinalis* distributions plotted per decade (1902 – year in top right corner of each map), where presence (in red) is plotted on top of absence (in blue). This is based on data from the current study and from A. Grau (pers. comm.) and on data from the literature listed in the Supplementary materials (below).



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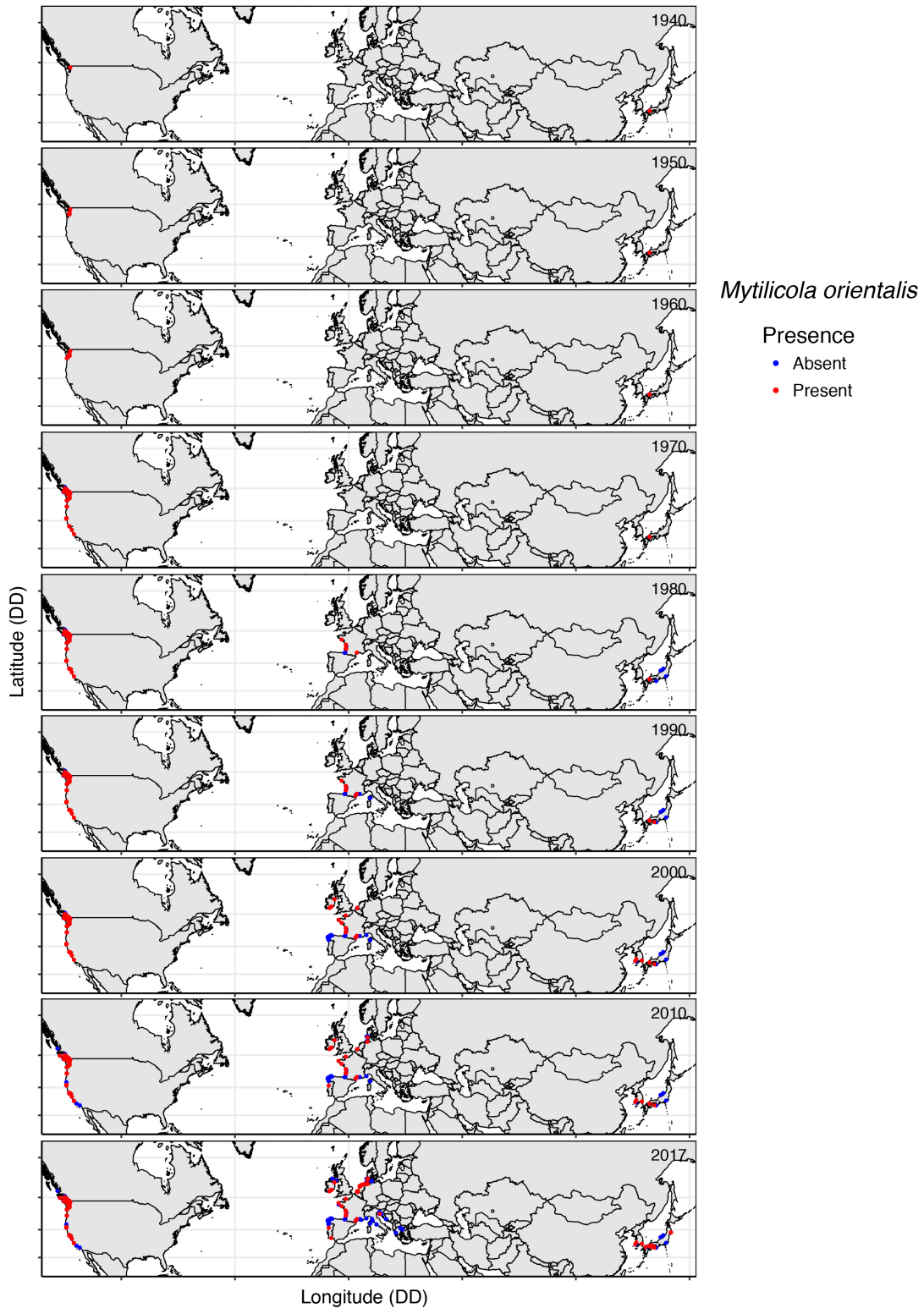
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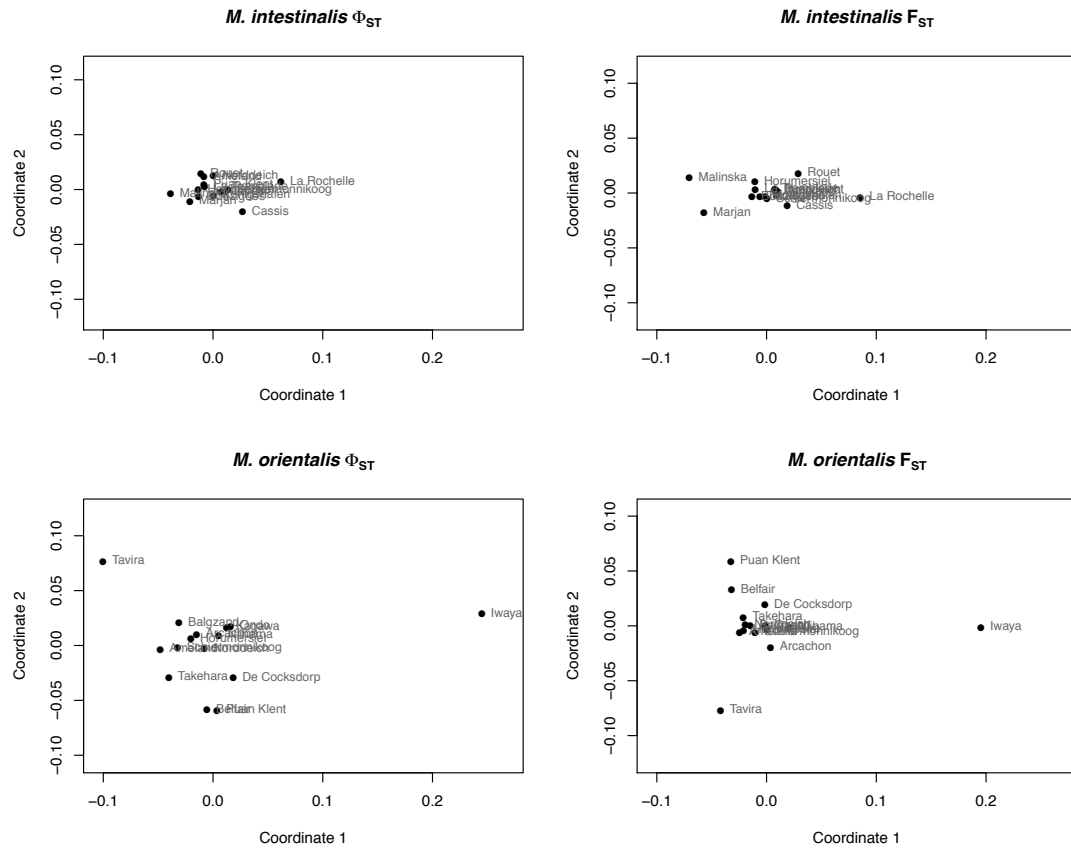


**Figure S2.2** Cumulative *Mytilicola orientalis* distributions plotted per decade (1935 – year in top right corner of each map), where presence (in red) is plotted on top of absence (in blue). This is based on data from the current study, from unpublished data (M. E. Feis, *Chapter 3*) and data from the literature listed in the Supplementary materials (below).

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**Figure S2.3** MDS plots of  $\Phi_{ST}$  (left column) and  $F_{ST}$  (right column) of *Mytilicola intestinalis* (upper row) and *Mytilicola orientalis* (lower row).

**Table S2.1** Haplotype frequencies for 483 bp fragment of cytochrome-c-oxidase 1 (COI) in *Mytilicola intestinalis*;  $\pi$ : nucleotide diversity;  $h$ : haplotype diversity; (SD): standard deviation between brackets.

	Mi1	Mi2	Mi3	Mi4	Mi5	Mi6	Mi7	Mi8	Mi9	Mi10	Mi11	Mi12	Mi13	Mi14	Mi15	Mi16	Mi17	Mi18	Sum	$\pi$ (SD)	$h$ (SD)
Balaruc, FRA	1																		1	0.000 (0.000)	1.00 (0.000)
Marjan Peninsula, CRO	27	1	1																29	0.000286 (0.00480)	0.1355 (0.0845)
Malinska, CRO	28	1		1															30	0.000276 (0.000471)	0.131 (0.0821)
Livorno, ITA	22	3	1		2			1		1					1				30	0.00119 (0.00119)	0.460 (0.109)
Cassis, FRA	22	1	4						3										30	0.00144 (0.00144)	0.448 (0.102)
Istres, FRA	25	1			1			1		2									30	0.00121 (0.00112)	0.308 (0.108)
Rouet, FRA	19	3		2	1	1	1	1		1	1	1							29	0.00163 (0.00136)	0.569 (0.106)
Bouzigues, FRA	26	1	1										1	1					30	0.000552 (0.000693)	0.223 (0.104)
La Rochelle, FRA	10		2						1							2	1		16	0.00267 (0.00198)	0.608 (0.130)
La Tremblade, FRA	19	1		1														1	22	0.000753 (0.000841)	0.260 (0.120)
Balgzand, NLD	4		1																5	0.000828 (0.00105)	0.4000 (0.237)
De Cocksdorp, NLD	5		1						1										7	0.00237 (0.00198)	0.524 (0.209)
Ameland, NLD	22	1	1	1	3	1	1	1											30	0.00131 (0.00118)	0.462 (0.110)
Schiermonnikoog, NLD	13		1		1			1											16	0.00100 (0.00102)	0.350 (0.148)
Horumerstel, DEU	18	2			1														21	0.000572 (0.000717)	0.267 (0.120)
Norddeich, DEU	33	1	2	2	2	2	4	4	1										45	0.00156 (0.00130)	0.458 (0.0898)
Puan Klent, DEU	22	1	1	2	2	2	1	1	1										30	0.00120 (0.00111)	0.464 (0.111)
Königshafen, DEU	19	2	2																23	0.000687 (0.000795)	0.316 (0.118)
Sum	335	19	18	8	1	13	2	8	2	9	1	1	1	1	1	2	1	1	424		

**Table S2.2** Haplotype frequencies for 476 bp fragment of cytochrome-c-oxidase 1 (COI) in *Mytilicola orientalis*;  $\pi$ : nucleotide diversity;  $h$ : haplotype diversity; (SD): standard deviation between brackets.

	Mo1	Mo2	Mo3	Mo4	Mo5	Mo6	Mo7	Mo8	Mo9	Mo10	Mo11	Mo12	Mo13	Mo14	Mo15	Mo16	Mo17	Mo18	Mo19	Mo20	Mo21	Mo22	Mo23	Mo24	Mo25	Mo26	Sum	$\pi$ (SD)	$h$ (SD)
Iwaya, JPN	4		3	3	3					14																	24	0.00550 (0.00340)	0.627 (0.092)
Ondo, JPN	8	3		11	4			3	1						1					1	1						33	0.00528 (0.00324)	0.820 (0.0425)
Takehara, JPN	6	3	2	12	1			2	1													1	1				29	0.00422 (0.00273)	0.788 (0.0609)
Niihama, JPN	8	1	7	10	1				1	2																	30	0.00468 (0.00295)	0.782 (0.0404)
Kagawa, JPN	6	1	2	8	3					1					1												21	0.00436 (0.00284)	0.776 (0.0597)
Ofunato, JPN			1																								1	0.000 (0.000)	1.00 (0.000)
Hiwasa, JPN							1																				1	0.000 (0.000)	0.000 (0.000)
Belfair, USA	3	2	1	10	2			1	1	1	1	1	1	1													22	0.00512 (0.00322)	0.784 (0.0828)
Harstine, USA			1																								1	0.000 (0.000)	1.00 (0.000)
Cortes, CAN	5		1	2																							8	0.00390 (0.00284)	0.607 (0.164)
Piper's Lagoon, CAN	2			3																							5	0.00378 (0.00304)	0.600 (0.175)
Fanny Bay, CAN					1	1																					2	0.00210 (0.00297)	1.00 (0.500)
Arachon, FRA	10	3	4	1	6	4	1	1	1																		31	0.00541 (0.00332)	0.839 (0.0408)
La Rochelle, FRA			1																								1	0.000 (0.000)	1.00 (0.000)
Tavira, PRT	16	2	2	6	6	1			3																1		31	0.00493 (0.00307)	0.699 (0.075)
Balgzand, NLD	9		3	12	2					1	1	1					1										29	0.00446 (0.00285)	0.739 (0.0565)
De Cocksdorp, NLD	5	2	3	11	6			1							1			1									30	0.00395 (0.00258)	0.807 (0.0481)
Ameland, NLD	10	2	3	11	2					2																	30	0.00437 (0.00280)	0.756 (0.0500)
Schiernminkoo g, NLD	9	1	4	10	3					2													1				30	0.00445 (0.00284)	0.791 (0.0447)
Horumstiel, DEU	10	6		11	3					1									1								32	0.00442 (0.00282)	0.762 (0.0402)
Norddeich, DEU	7	2	2	10	5					1									1								28	0.00421 (0.00272)	0.794 (0.0470)
Puan Klent, DEU	3	2	2	13	1					1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	25	0.00366 (0.00245)	0.723 (0.0918)
Sum	121	33	39	1	150	40	1	1	1	8	12	1	1	1	22	1	1	1	1	1	2	1	1	1	1	1	444		

**Table S2.3** Pairwise population comparisons (below diagonal) for *Mytilicola intestinalis* for all locations ( $N \geq 15$ ), based on partial cytochrome-c-oxidase 1 (COI) sequences. None of the values are significantly different from 0 after Bonferroni correction (corrected p-level  $0.05/105 = 0.000476$ ). P-values are mentioned in the upper half of the table (above diagonal).

	Marjan	Malinska	Livorno	Cassis	Istres	Rouet	Bouzigues	La Rochelle	La Tremblade	Ameland	Schiermonnikoog	Horumersiel	Norddeich	Puan Klent	Königshafen
Marjan	-	0.86972	0.58202	0.08336	0.47589	0.12613	0.99990	0.01030	0.63944	0.16642	0.20345	0.46184	0.08940	0.29245	0.53411
Malinska	-0.01723	-	0.35224	0.02485	0.48649	0.12405	0.99990	0.00129	0.85110	0.10524	0.07732	0.44817	0.08613	0.41620	0.25542
Livorno	-0.00075	0.00643	-	0.25394	0.77586	0.68221	0.57044	0.09177	0.50183	0.89021	0.62548	0.99990	0.35907	0.72834	0.77210
Cassis	0.04257	0.06074	0.01063	-	0.42085	0.00832	0.08366	0.66429	0.08930	0.02822	0.13523	0.09266	0.04208	0.02554	0.24671
Istres	0.01171	0.01283	-0.01372	-0.00116	-	0.10058	0.49282	0.28344	0.40204	0.27829	0.24423	0.47807	0.17573	0.41263	0.26483
Rouet	0.02154	0.01364	-0.01100	0.05155	0.01851	-	0.05366	0.00277	0.43857	0.57291	0.42055	0.82427	0.23423	0.59954	0.35214
Bouzigues	-0.02351	-0.01124	0.00067	0.03969	0.00829	0.02026	-	0.00980	0.74210	0.17097	0.20998	0.53292	0.07296	0.28710	0.53599
La Rochelle	0.08404	0.10039	0.03032	-0.02622	0.01087	0.06644	0.07315	-	0.00376	0.00990	0.20691	0.00535	0.04406	0.01020	0.07742
La Tremblade	-0.00804	-0.02057	-0.00314	0.04155	0.00503	-0.00245	-0.00797	0.06224	-	0.20770	0.15296	0.59261	0.14909	0.49936	0.53807
Ameland	0.02229	0.02945	-0.01270	0.04734	0.01557	-0.00780	0.02049	0.06454	0.01580	-	0.96644	0.71023	0.78933	0.93070	0.28859
Schiermonnikoog	0.03356	0.05091	-0.00983	0.02711	0.00964	0.00309	0.02126	0.03729	0.02218	-0.03460	-	0.38105	0.98475	0.96010	0.23602
Horumersiel	-0.00452	-0.00286	-0.02934	0.04382	-0.00027	-0.02097	-0.00564	0.07079	-0.01260	-0.01487	0.00755	-	0.26730	0.61291	0.61133
Norddeich	0.02800	0.02928	0.00309	0.03819	0.01424	0.00824	0.02785	0.05130	0.01964	-0.01255	-0.03627	0.00805	-	0.90664	0.12444
Puan Klent	0.01179	0.00702	-0.00627	0.04310	0.00846	-0.00806	0.00851	0.06058	-0.00254	-0.01718	-0.03253	-0.00915	-0.01707	-	0.30928
Königshafen	-0.01453	0.01411	-0.01532	0.01650	0.01588	0.00260	-0.01150	0.04907	0.00009	0.00990	0.01711	-0.01332	0.02388	0.00882	-

**Table S2.4** Pairwise population comparisons with conventional F-statistics (below diagonal) for *Mytilicola intestinalis* for all locations ( $N \geq 15$ ), based on partial cytochrome-c-oxidase I (COI) sequences. None of the values are significantly different from 0 after Bonferroni correction (corrected p-level  $0.05/105 = 0.000476$ ). P-values are mentioned in the upper half of the table (above diagonal).

	Marjan	Malinska	Livorno	Cassis	Istres	Rouet	Bouzigues	La Rochelle	La Tremblade	Ameland	Schiermonnikoog	Horumeriel	Norddeich	Puan Klent	Königshafen
Marjan	-	0.99990	0.08336	0.06267	0.36887	0.01683	0.87130	0.01158	0.78260	0.07019	0.33017	0.46025	0.05128	0.08623	0.42273
Malinska	-0.02590	-	0.06386	0.01643	0.36521	0.01733	0.80022	0.00475	0.71260	0.03930	0.32700	0.46283	0.04010	0.07821	0.22384
Livorno	0.04705	0.05387	-	0.55658	0.58608	0.69508	0.28661	0.30512	0.29957	0.96099	0.70211	0.58499	0.62608	0.91912	0.60925
Cassis	0.06190	0.07884	-0.00645	-	0.29849	0.18998	0.14276	0.52341	0.17533	0.36056	0.50312	0.18681	0.35353	0.39432	0.48787
Istres	0.00504	0.00710	-0.00734	0.00774	-	0.11949	0.83210	0.09346	0.83190	0.42402	0.87061	0.78022	0.30165	0.52124	0.57014
Rouet	0.08857	0.08707	-0.01464	0.01464	0.02475	-	0.06069	0.27730	0.16979	0.59054	0.35798	0.23443	0.51074	0.77774	0.21117
Bouzigues	-0.01791	-0.01028	0.01022	0.02317	-0.01371	0.04224	-	0.03742	0.99990	0.21265	0.84655	0.84249	0.15662	0.26334	0.64162
La Rochelle	0.13942	0.15553	0.00697	-0.01446	0.04931	0.00416	0.07164	-	0.06257	0.29255	0.36878	0.05762	0.23800	0.30809	0.17196
La Tremblade	-0.01370	-0.02030	0.00300	0.02632	-0.01914	0.02294	-0.02511	0.06735	-	0.24354	0.67993	0.84992	0.26968	0.42402	0.69775
Ameland	0.05032	0.05690	-0.02174	0.00327	-0.00159	-0.01003	0.01312	0.00868	0.00788	-	0.93753	0.45095	0.84101	0.98446	0.41135
Schiermonnikoog	0.00811	0.02102	-0.02080	-0.01163	-0.02403	0.0015	-0.02230	0.01075	-0.01984	-0.03405	-	0.79091	0.94694	0.91694	0.75438
Horumeriel	-0.00796	-0.00559	-0.01572	0.02466	-0.02357	0.01574	-0.02255	0.06644	-0.03049	-0.00605	-0.02405	-	0.25552	0.43916	0.73141
Norddeich	0.04708	0.04982	-0.00846	0.00078	0.00383	-0.00602	0.01530	0.00970	0.00667	-0.01593	-0.03100	0.00652	-	0.97842	0.37125
Puan Klent	0.04670	0.04651	-0.01927	0.00076	-0.00458	-0.01688	0.00994	0.00638	-0.00318	-0.02432	-0.03167	-0.00492	-0.02181	-	0.48480
Königshafen	-0.00056	0.01598	-0.01563	-0.00828	-0.01099	0.013	-0.02170	0.02993	-0.01914	-0.00374	-0.02934	-0.02774	-0.00002	-0.00665	-



**Table S2.5** Pairwise population comparisons (below diagonal) for *Mytilicola orientalis* for all locations (N ≥ 15), based on partial cytochrome-c-oxidase 1 (COI) sequences. Values in bold are significantly different from 0 after Bonferroni correction (corrected p-level 0.05/105 = 0.000476). P-values are mentioned in the upper half of the table (above diagonal).

	Iwaya	Ondo	Takehara	Niihama	Kagawa	Belfair	Arcachon	Tavira	Balgzand	De Cocksdorp	Ameland	Schiermonnikoog	Horumerstel	Norddeich	Puan Klent
Iwaya	-	0.00010	0.00000	0.00040	0.00129	0.00010	0.00000	0.00000	0.00010	0.00020	0.00000	0.00000	0.00000	0.00000	0.00000
Ondo	<b>0.21472</b>	-	0.54351	0.30720	0.80992	0.10316	0.64598	0.03871	0.28215	0.41758	0.49114	0.52500	0.60638	0.77250	0.29601
Takehara	<b>0.28984</b>	-0.00914	-	0.38551	0.40511	0.24889	0.44827	0.01881	0.10870	0.39867	0.41630	0.56252	0.26750	0.51797	0.72300
Niihama	<b>0.23132</b>	0.00288	-0.00138	-	0.79982	0.07920	0.69399	0.03930	0.40055	0.35016	0.58766	0.84269	0.23008	0.52470	0.23018
Kagawa	0.22019	-0.02347	-0.00603	-0.02662	-	0.17266	0.91793	0.09266	0.67825	0.62687	0.72448	0.88635	0.72933	0.99515	0.28512
Belfair	<b>0.26448</b>	0.03027	0.01151	0.03745	0.02630	-	0.07306	0.00238	0.02455	0.23661	0.05752	0.09920	0.06752	0.17315	0.38323
Arcachon	<b>0.25426</b>	-0.01257	-0.00547	-0.01590	-0.02924	0.03954	-	0.16503	0.73250	0.25641	0.98852	0.98921	0.63231	0.76171	0.13444
Tavira	<b>0.34408</b>	0.05878	0.09423	0.06798	0.05197	0.15717	0.02343	-	0.21236	0.00297	0.21612	0.10435	0.08098	0.02930	0.00446
Balgzand	<b>0.27312</b>	0.00562	0.02793	-0.00511	-0.02307	0.07534	-0.01820	0.00972	-	0.07079	0.77299	0.64330	0.37986	0.33165	0.05108
De Cocksdorp	<b>0.22833</b>	-0.00317	-0.00258	0.00182	-0.01600	0.01234	0.00917	0.14485	0.04530	-	0.12524	0.24186	0.19335	0.78170	0.87298
Ameland	<b>0.29231</b>	-0.00733	-0.00364	-0.01286	-0.02374	0.05192	-0.02780	0.01504	-0.02211	0.02707	-	0.99990	0.49500	0.53747	0.11078
Schiermonnikoog	<b>0.27568</b>	-0.00916	-0.01038	-0.02205	-0.02943	0.03976	-0.02715	0.03632	-0.01740	0.00881	-0.03053	-	0.41214	0.75725	0.18860
Horumerstel	<b>0.26147</b>	-0.01226	0.00745	0.01074	-0.02401	0.04535	-0.01472	0.04482	-0.00192	0.01684	-0.01055	-0.00480	-	0.55787	0.09039
Norddeich	<b>0.24957</b>	-0.01836	-0.00875	-0.01023	-0.03610	0.02244	-0.01899	0.07688	0.00226	-0.01995	-0.01184	-0.02033	-0.01404	-	0.39442
Puan Klent	<b>0.25493</b>	0.00562	-0.01608	0.01176	0.00891	0.00292	0.02503	0.16660	0.07160	-0.02253	0.03825	0.01993	0.03638	-0.00248	-

**Table S2.6** Pairwise population comparisons with conventional F-statistics (below diagonal) for *Mytilicola orientalis* for all locations ( $N \geq 15$ ), based on partial cytochrome-c-oxidase I (COI) sequences. Values in bold are significantly different from 0 after Bonferroni correction (corrected p-level  $0.05/105 = 0.000476$ ). P-values are mentioned in the upper half of the table (above diagonal).

	Iwaya	Ondo	Takehara	Niihama	Kagawa	Belfair	Arcachon	Tavira	Balgzand	De Cocksdorp	Ameland	Schiermommikoog	Horumerstel	Norddeich	Puan Klent
Iwaya	-	0.00000	0.00000	0.00000	0.00040	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
Ondo	<b>0.19202</b>	-	0.82170	0.16771	0.86338	0.54381	0.38293	0.03455	0.42986	0.72468	0.60766	0.58004	0.71706	0.87486	0.24720
Takehara	<b>0.21599</b>	-0.01664	-	0.37036	0.77735	0.89219	0.16474	0.01970	0.65300	0.56796	0.70745	0.60737	0.49470	0.66439	0.84368
Niihama	<b>0.16504</b>	0.01624	0.00021	-	0.69468	0.19463	0.36056	0.04613	0.62786	0.32135	0.69894	0.85229	0.12425	0.37937	0.17177
Kagawa	<b>0.19066</b>	-0.02274	-0.02010	-0.01810	-	0.68657	0.58885	0.09791	0.97832	0.91466	0.94426	0.96674	0.66766	0.99990	0.40956
Belfair	<b>0.23027</b>	-0.00744	-0.02325	0.01620	-0.01781	-	0.07079	0.00495	0.43233	0.74795	0.35541	0.34967	0.32838	0.64172	0.97099
Arcachon	<b>0.18733</b>	0.00065	0.01700	0.00202	-0.0114	0.03562	-	0.24017	0.23493	0.28670	0.49084	0.65726	0.32828	0.52797	0.01455
Tavira	<b>0.24658</b>	0.04757	0.06796	0.04657	0.03698	0.10844	0.01093	-	0.05930	0.00594	0.20533	0.15177	0.07920	0.04584	0.00168
Balgzand	<b>0.20750</b>	-0.00287	-0.01125	-0.01293	-0.03421	-0.00313	0.01080	0.04260	-	0.41788	0.91229	0.86942	0.32512	0.67647	0.29472
De Cocksdorp	<b>0.19559</b>	-0.01304	-0.00853	0.00580	-0.02839	-0.01729	0.00749	0.08844	-0.00218	-	0.41144	0.58212	0.28888	0.96268	0.39739
Ameland	<b>0.21723</b>	-0.00957	-0.01487	-0.01328	-0.03137	0.00188	-0.00505	0.01362	-0.02431	0.00078	-	0.99990	0.58559	0.81249	0.21830
Schiermommikoog	<b>0.20231</b>	-0.00812	-0.01009	-0.01958	-0.03273	0.00275	-0.01167	0.02028	-0.02219	-0.00837	-0.02989	-	0.40115	0.89011	0.18662
Horumerstel	<b>0.21377</b>	-0.01385	-0.00600	0.02378	-0.01733	0.00375	0.00256	0.03500	0.00357	0.00651	-0.01088	-0.00049	-	0.59727	0.12345
Norddeich	<b>0.21300</b>	-0.01996	-0.01297	0.00061	-0.03724	-0.01420	-0.00640	0.04774	-0.01501	-0.02632	-0.02032	-0.02380	-0.01150	-	0.31225
Puan Klent	<b>0.23398</b>	0.00918	-0.02052	0.01990	-0.00132	-0.02811	0.06490	0.13514	0.00544	-0.00009	0.01426	0.01728	0.02723	0.00551	-





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Do invasions create competitors? Co-infections of  
specialist *Mytilicola intestinalis* vs. generalist  
*Mytilicola orientalis* in blue mussel hosts

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*Manuscript in preparation*

## Abstract

Specialist parasites should have a competitive advantage over generalists due to costs of generalism. Generalist and specialist taxa should therefore not occupy the same niche over extended periods of time. Biological invasions can however create new competitive situations and here we used the consecutive invasions of two congeneric parasitic copepods to test the competitive balance between generalists and specialists shortly after invasion. First the specialist *Mytilicola intestinalis* and later the generalist *Mytilicola orientalis* invaded into the North Atlantic, where they now infect blue mussels *Mytilus edulis* as a shared host. Recently, *M. orientalis* has become more abundant, suggesting that the generalist might benefit from the specialist or even has a competitive advantage in the shared host. Here, we now investigate the competitive or facilitative interactions between these two parasites in experimental co-infections in different host backgrounds that naturally either experience infections by both (Texel, southern Wadden Sea, the Netherlands), only one species (Sylt, northern Wadden Sea, Germany), or were naïve to both species (Kiel, Baltic Sea, Germany). In general, the established specialist *M. intestinalis* had higher infection rates than the newly invading generalist *M. orientalis*, showing there is a cost to generalism. Within co-infections, neither infection rates nor parasite growth of *M. intestinalis* and *M. orientalis* were different from those expected from single-species infections, indicating that there was no direct competition between or superiority of either species. We did not find a cumulative effect of parasite infection on the host body condition in co-infections. Instead, their condition may resemble that of hosts infected by *M. orientalis* only the most, but this could not be demonstrated significantly and further work is needed. The lack of competitive or facilitative interactions between the two invasive parasites suggests that the successful invasion of *M. orientalis* relies on the spillover of *M. orientalis* from Pacific oysters to several different native host species, and furthermore suggests that the complex multispecies ecological interactions between native hosts and invasive *Mytilicola orientalis* can compensate for the cost of generalism of this parasite.

**Keywords:** multiple infections; concurrent infection; biological invasions; host–parasite interactions; parasite-parasite interactions; invertebrates

## Introduction

Invasive species often have negative impacts on the native community (Lowe *et al.* 2000; meta-analysis by Jackson 2015). In the case of invasive parasites infecting native hosts, negative effects are obvious for the hosts, but less clear for the established parasites infecting the same hosts. A native parasite could experience competition with a newly invading parasite when utilizing the same niche within a shared host.

Infections by multiple parasites are common in animals in general (Cox 2001; Lello *et al.* 2004; Petney & Andrews 1998; Warne *et al.* 2016) and a multitude of interspecific interactions can exist between concurrently infecting parasites. These range from no interaction (Morand *et al.* 2002 and references therein) to mutual antagonism (e.g., Collier & Hunter 2001; Wilson 1916), facilitation (e.g., Lusso *et al.* 1991; Schall & Bromwich 1994) and cooperation (e.g., West & Buckling 2003). Strong interspecific competition has been shown to lead to changes in the occupied niches of parasites and competitive exclusion (e.g., Andrews *et al.* 1982; Chappell 1969; Paperna 1964). Three forms of competition can be distinguished. In exploitation competition, an indirect interaction, parasites compete over shared resources, in which a higher consumption leads to a higher fitness (Dobson 1985; Miller 1967), for an example, see for instance Ruiz Daniels *et al.* (2012). In interference competition, parasites directly inhibit the growth, reproduction or transmission of the other (Dobson 1985; Miller 1967), either mechanically or with chemicals (see for instance Bashey *et al.* 2013; Collier & Hunter 2001). In apparent competition, the presence of one parasite species has a negative effect on the other's population density indirectly (Holt 1977), for instance by stimulating a host response that acts against both parasite species (e.g., Lysenko *et al.* 2005). Similarly, hosts can mediate interactions between concurrently infecting parasites (e.g., Bush & Malenke 2008). The ecological and evolutionary consequences of a co-infection for the host, but also for the parasitic agents themselves, can greatly differ depending on the nature of parasite–parasite interactions as well as host–parasite interactions (Mideo 2009).

Successful invaders often have broad environmental niches (e.g., *Mya arenaria*, Strasser 1999; *Carcinus maenas*, Leignel *et al.* 2014) and physiological niches (Higgins & Richardson 2014), and can therefore be characterised as generalists. A generalist as the “jack of all trades, master of none” (MacArthur 1972) has a reduced

efficiency compared to a competing specialist, for it needs to keep track of more environmental information to exploit the optimal conditions in each local environment (Dall & Cuthill 1997) – in the case of generalist parasites, more immune systems of different host species have to be dealt with. The cost of generalism is thought to lead to the evolution of specificity and specialism (Futuyma & Moreno 1988) and specialist parasites should therefore have an advantage over competing generalist species and outcompete generalists in their only host. For example, specialist canestriid mites infecting two species of lucanid beetles outcompeted generalist mites, demonstrating clear superiority of the specialist (Okabe *et al.* 2012). Also generalist parasites may have a greater sensitivity to competition than specialists (Dawson *et al.*, 2000). Therefore, specialists and generalists should not co-occur in the same niche for extended periods of time. Invasions, however, can create competitive situations and experiments using invasive parasites can therefore shed light on the early stages of competitive interactions.

In this study we focus on two congeneric species of parasitic copepods that both invaded the North Sea where they now infect the intestinal tract of blue mussels *Mytilus edulis* as a new host (Elsner *et al.* 2010; Goedknecht *et al.* 2017a; Pogoda *et al.* 2012). Both species also differ in their degree of host specificity. *Mytilicola intestinalis* is specialised on mytilid mussels and first invaded mussel beds the Wadden Sea in the late 1930's (Caspers 1939) and is now distributed throughout the whole Wadden Sea (Dethlefsen 1972; Drinkwaard 1993). In lab settings *M. intestinalis* readily infects other species than *Mytilus edulis* including flat oysters *Ostrea edulis*, *Cerastoderma edule*, *Crepidula fornicata* and *Paphia pullastra* (Hepper 1953), but when given the choice between flat oysters and mussels, *M. intestinalis* only infected mussels (Bolster 1954; Hepper 1956). The generalist *Mytilicola orientalis* co-invaded European mussel beds with the Pacific oyster *Magellana gigas* (His 1977a). Its first detection in the Wadden Sea was in 2007 (Elsner *et al.* 2010; Pogoda *et al.* 2012). Within the Wadden Sea and southern North Sea area the host range of *M. intestinalis* is narrow and includes only blue mussels *Mytilus edulis*, while the host range of *M. orientalis* is considerably broader and includes Pacific oysters *M. gigas*, blue mussels *M. edulis*, common cockles *Cerastoderma edule* and Baltic tellins *Limecola balthica* (Goedknecht *et al.* 2017a). Since both parasites use the same space in the mussel gut, we hypothesise that there



are competitive interactions between the parasites, and we question if competitive superiority is contributing to the invasion success of *M. orientalis*.

Therefore our aim here is to elucidate whether competitive ability of generalist and specialist parasites contribute to their invasion success. We do this through testing whether there is superiority of specialist *M. intestinalis* over generalist *M. orientalis* in their shared host, and if there is a cumulative effect of co-infections on the condition of their shared host at similar total parasite loads.

## **Materials and Methods**

### **Field data from Königshafen**

To document background infection levels of both *Mytilicola* species in the wild, we collected invasive Pacific oysters *M. gigas* and native blue mussels *M. edulis* in the oyssel reef in Königshafen, Sylt, Germany between 2013 and 2016. The bivalves were dissected under a stereomicroscope to look for *Mytilicola* infections. Species determination of the parasites was done based on morphology. Note that *M. orientalis* was absent from this location in 2008 (Elsner et al. 2010).

### **Oyster infections**

To test specificity of *M. intestinalis* we collected 16 Pacific oysters *M. gigas* ranging from 4.3 to 12.5 cm from the “oyssel reef” (a multilayered, mixed bed of mussels and oysters, *sensu* Reise et al. 2017b) in Königshafen on Sylt, Germany in spring 2014. Mussels from the oyssel reef in Puan Klent on Sylt, Germany, were the source of *M. intestinalis* for the oyster experiment. Copepodites were collected in a similar fashion as described in Feis et al. (2016) and above. In our experiment the 16 oysters were exposed to 25 *M. intestinalis* copepodites each. For infections, oysters were put in separate containers with filtered seawater and copepodites were pipetted into the seawater. Seawater of each container was checked for remaining copepodites after 2-4 days and only a maximum of one copepodite was still found in the water. Oysters were then pooled into one tank and were connected to the flow-through system described in Feis et al. (2016). Pacific oysters were dissected under a stereomicroscope after 133 days to count parasites.

### **Superiority experiment**

The experimental procedures of this study, including collection, treatment against previous infections of *Mytilicola* spp., infection procedures, feeding, dissection, and the measurements that were taken are the same as described in Feis *et al.* (2016) (Chapter 4). In short, we collected mussels of similar shell length (3.5–5.0 cm) in three locations that differ in terms of parasite occurrence. These included the Dutch Wadden island Texel (Vlakte van Kerken), where *M. intestinalis* and *M. orientalis* co-occur, the German Wadden island Sylt (Königshafen), where only *M. intestinalis* occurred in mussels at time of sampling, and in the Kiel harbour, where both parasite species were absent. For infection, we collected egg sacs from individual gravid female parasites. We took egg sacks from Texel *M. orientalis*, as it was not found in mussels from the Königshafen on Sylt in 2014, and *M. intestinalis* egg sacs from Sylt. Once eggs hatched and larvae reached the infective copepodite stage, we infected individual mussels by adding 24 copepodites to a small Kautex bottle filled with seawater. For the single-parasite treatments, we added 24 *M. intestinalis* or *M. orientalis*. We used a substitution design for the co-infection treatment by administering 12 *M. intestinalis* and 12 *M. orientalis* copepodites to keep the same starting density in all infection treatments. Mussels in the control treatments were handled in the same way as those in the infection treatments, but were not exposed to any copepodites. We used 15 mussels in each treatment group. We counted non-ingested parasites after 24 hours to calculate exposure dose for each mussel. Mussels were dissected after 80 days and we measured host length, host weight and parasite length, we counted parasite numbers and calculated infection rate. Parasite species in the co-infection treatment was determined using a TaqMan® assay (Goedknecht *et al.* 2018).

### **Data handling and statistical analyses**

All analyses were performed in the R statistical environment Version 3.4.3 (R Core Team 2017). Host length was normally distributed (Shapiro-Wilk test,  $p = 0.0595$ ) and had similar distribution of length across all treatments, as we found no significant differences in host length between host source (ANOVA,  $df = 2$ ;  $F = 0.9107$ ;  $p = 0.405$ ), parasite source (levels: control, *M. intestinalis*, *M. orientalis* and concurrent) (ANOVA,  $d.f. = 3$ ;  $F = 1.3219$ ;  $p = 0.270$ ) or host source  $\times$  parasite source (ANOVA,

d.f. = 6;  $F = 0.7527$ ;  $p = 0.608$ ), confirming that we selected hosts of similar lengths for our experiments.

Host survival was analysed with a binomial generalised linear model (GLM) estimating the effects of host source, infection treatment (levels: control, infected with *M. intestinalis*, infected with *M. orientalis* and concurrently infected) and the interaction between host source and infection treatment. We used a stepwise selection procedure based on Akaike's information criterion (AIC) to optimise this model.

Infection rates were calculated by dividing the infection intensity by the exposure dose after controlling for parasites that were not ingested after 24h. The exposure dose for co-infections was rounded up from 0.5 when only 1 parasite was not ingested in order to have integers for the binomial model. Since only one parasite species can be found in the single infections we could not fit an interaction term between parasite species and treatment (levels: single and co-infection). Therefore we combined both factors into one factor with four levels. We fitted a binomial generalised linear mixed model (GLMM) testing successful against failed infections as a function of host source and a combined factor of species and treatment, plus the interaction term, with mussel ID as a random factor (glmer function of the nlme package). We tested this model in a type III Wald chisquare Analysis of Deviance, which tests for the presence of a main effect after accounting for the other main effects and interactions (car package). To test whether there were differences in infection rates of *M. intestinalis* and *M. orientalis* within co-infections compared to single infections, we used a post hoc Tukey test on the combined factor (multcomp package).

Dry weight of the mussel flesh was used as a measure for the host condition and was normally distributed after applying a log transformation (Shapiro-Wilk normality test,  $p = 0.1174$ ). We only included mussels from the co-infection treatment that were actually infected by both species. There was a tendency that these mussels were only infected by *M. intestinalis* (N = 4, 3 and 0 for Texel, Sylt and Kiel, respectively) except one mussel from Texel, which was only infected by *M. orientalis*. Because weight increases with body size, we corrected for host length by fitting it as the first term in the model. One mussel from Kiel infected with *M. intestinalis* was excluded from the dataset because the dry weight was more than 3 standard deviations larger than the mean dry weight of that group (mean = 0.2237 g; sd = 0.2035; outlier = 0.9165 g), which was most likely due to a measurement error. Our sequential model

fitting strategy for host condition was as follows. To test for the effect of infection treatment (levels: control, *M. intestinalis*, *M. orientalis*, and co-infection) on the host condition, we modelled log-transformed dry weight in a linear model as a function of host length, infection intensity, host source, infection treatment and all interactions between the terms infection intensity, host source, and infection treatment (terms in the full model). Because of the large variation in dry weight within the control treatment on Texel, we also fitted the same dry weight model with only the parasite treatments (*M. intestinalis*, *M. orientalis* and co-infections). To look at the effects of the parasite treatments without the largest influencing factor on host condition, we split up the dataset into the different host sources and ran the same dry weight model for Texel, Sylt and Kiel hosts separately. To optimise all dry weight models, we used a stepwise selection procedure based on Akaike's information criterion (AIC).

Length of individual parasites was taken as a measure of growth rate. From this dataset, all broken or damaged parasites were omitted from the analyses because it was not possible to get a reliable length measurement for these individuals. Three mussels from Kiel and one mussel from Texel from the co-infection treatment were omitted in the parasite length analyses because we could not determine species affiliation for all parasites reliably. First, parasite length (log-transformed) was analysed with a GLMM as a function of sex, host source, a combined factor with species and treatment, and the interaction between the combined factor and host source, with mussel ID as a random factor (lmer function of the nlme package). Second, we split the dataset to separate any effects of sex on the analyses, to arrive to two GLMMs for female and male parasite length. Here, we analysed parasite length of female or male parasites as a function of host source, a combined factor with species and treatment, and the interaction between the combined factor and host source, with mussel ID as a random factor. We tested these models in a type III Wald chisquare Analysis of Deviance (car package). To test whether there were differences in parasite length between *M. intestinalis*, *M. orientalis* (species), single and co-infections (treatment), we used a post hoc Tukey test on the combined factor (multcomp package). Because of a significant interaction between host source and the combined factor in the parasite length and female length models, we substituted host source and the combined factor in new parasite length models with a combined factor of all three factors (species, treatment and host source), so that we could distinguish which levels were significantly different in a post hoc Tukey test afterwards.

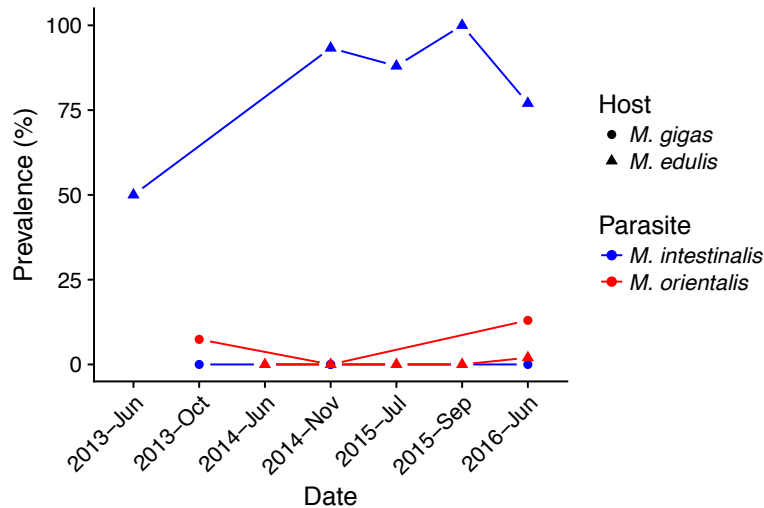
The number of gravid females of each species compared to the number of non-gravid females was used as a measure of parasite reproduction status. Co-infections were split by species. Parasite reproduction was analysed with a binomial GLMM testing number of gravid females against number of non-gravid females as a function of treatment with mussel ID as a random factor. It was not possible to fit host source and parasite species in as factors in this model, because of the absence of gravid *M. intestinalis* in general, and absence of gravid *M. orientalis* in Sylt and Texel mussels.

## Results

### Oyster infections and Königshafen field data

During the period 2013 – 2016, prevalence and mean intensity of *M. intestinalis* was high in mussels of Königshafen (77% – 100% and 4.54 – 6.89 respectively, Figure 3.1, Table 3.1). The highest intensity by *M. intestinalis* within one mussel was 29 specimens. Even though a single specimen of *M. orientalis* was twice found in Pacific oysters in October 2013, it was not found in mussels until June 2016, when 2% of the dissected mussels were infected. The infection intensity of *M. orientalis* was very low in mussels ( $1 \pm 0$ ) and oysters ( $1.62 \pm 0.65$ , Table 3.1), but nevertheless could signify the spread of *M. orientalis* into this area with the onset of competition between the two parasite species and nicely sets the stage for our experimental infections.

*Mytilicola intestinalis* on the other hand was not found in any of the Pacific oysters dissected between 2013 and 2016 (Figure 3.1, Table 3.1). Our infection experiment supported this, because only one oyster of 12 surviving oysters in the infected experiment group harboured a single *M. intestinalis* specimen. This shows that the parasites we selected were in principle viable and that Pacific oysters are not compatible hosts for *M. intestinalis*.



**Figure 3.1** Prevalence of *Mytilicola intestinalis* (blue) and *Mytilicola orientalis* (red) in *Magallana gigas* (bullets) and *Mytilus edulis* (triangles). Data are from the natural oyster reef in Königshafen, Sylt, Germany during the period 2013-2016. For more details, see Table 3.1.

**Table 3.1** Field data on prevalence (%) and mean infection intensity of *Mytilicola intestinalis* and *Mytilicola orientalis* in blue mussels *Mytilus edulis* and Pacific oysters *Magallana gigas* from the natural oyster reef in Königshafen, Sylt, Germany, collected at irregular intervals during the period 2013 – 2016. Sample size is denoted with N; host length given in cm (mean ± SD). Note that this is currently the most northern record of *M. orientalis* in mussels and oysters in continental Europe. In 2008, *M. orientalis* was still absent from this location (Elsner et al. 2010).

Date	Host	N	Host length	<i>M. intestinalis</i>		<i>M. orientalis</i>	
				Prevalence	Intensity	Prevalence	Intensity
10/10/2013	<i>M. gigas</i>	27	6.91 ± 2.42	0	-	7.4	1
29/06/2014	<i>M. edulis</i>	>100*	NA	NA	NA	0	-
17/11/2014	<i>M. gigas</i>	15	8.46 ± 2.58	0	-	0	-
17/11/2014	<i>M. edulis</i>	30	4.21 ± 1.08	93.3	6.89	0	-
21/07/2015	<i>M. edulis</i>	25	4.79 ± 0.76	88	4.72	0	-
08/09/2015	<i>M. edulis</i>	30	4.62 ± 0.57	100	5.13	0	-
10/06/2016	<i>M. gigas</i>	100	8.71 ± 2.56	0	-	13	1.62
10/06/2016	<i>M. edulis</i>	100	4.55 ± 0.90	77	4.54	2	1.5

\* These mussels were dissected to collect *M. intestinalis* egg sacks for the experiments described in Chapters 3, 4 and 5. While number of dissected mussels, and the prevalence and intensity of *M. intestinalis* were not noted, we noticed the absence of *M. orientalis*.

### Superiority experiment

All mussels in the control group were free of parasites after 80 days. Hence, the infections we found in the experimentally infected mussels did not come from any cross-infections between individual hosts or from residual infections from the field, but were due to our experimental exposure. Twenty-eight mussels died throughout the

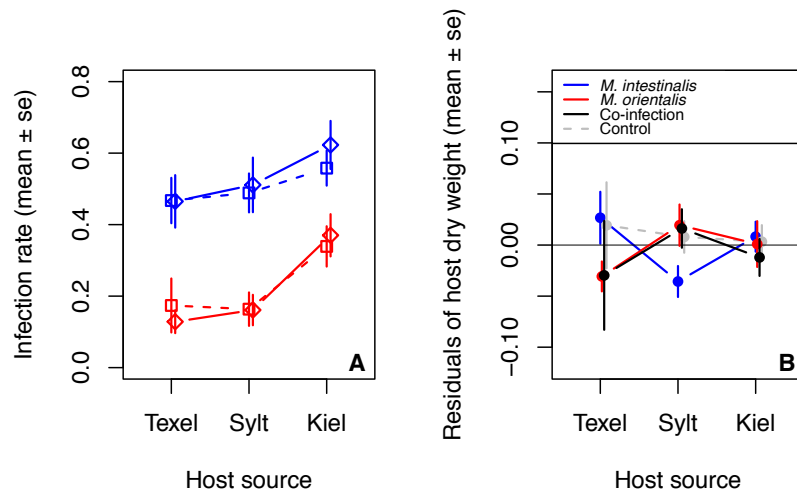
experiment. Mortality was not evenly distributed between all experimental groups (13 from Texel and 12 from Sylt and 3 from Kiel) and, accordingly, we found a significant difference in host survival between host sources (Analysis of Deviance;  $Deviance_{\text{host source}} = 8.4262$ , d.f. = 2, 173,  $p = 0.015$ ), but not between treatments ( $Deviance_{\text{infection treatment}} = 1.6081$ , d.f. = 3, 175,  $p = 0.651$ ) or the interaction term host source  $\times$  treatment ( $Deviance_{\text{host source} \times \text{infection treatment}} = 5.1772$ , d.f. = 6, 167,  $p = 0.521$ ). Species identity of parasites from the treatments with single species infections was confirmed based on morphology and species affiliation of parasites in the co-infection treatment was identified molecularly. Both methods confirmed a successful selection of egg sacs from gravid females of the two different species: all *M. orientalis* in the single-species treatment were identified as *M. orientalis*, all *M. intestinalis* as *M. intestinalis* and none of the co-infected mussels had more than 12 specimen of either species.

### Infection rates

In our experiment, the specialist *M. intestinalis* had a higher infection rate than the generalist *M. orientalis* (mean infection rate  $\pm$  SE =  $0.507 \pm 0.032$  and  $0.238 \pm 0.037$  for single species infections of *M. intestinalis* and *M. orientalis* respectively, Table 3.2, Figure 3.2A). Only the differences between species was driving the significance of the species by treatment combination, indicating that the infection rates of *M. intestinalis* and *M. orientalis* within co-infections were not significantly different from infections rates of either species in single infections (Tukey's HSD posthoc test: 3 out of 4 between species comparisons were significant with  $p$ -values  $< 0.05$ , while both within species comparisons were not significant, Figure 3.2A).

**Table 3.2.** Analysis of deviance tables for the binomial GLMM of infection rate (type III Wald chisquare test). The factors parasite species (divided into *M. intestinalis* and *M. orientalis*) and treatment (single infections and co-infections) are combined. Factors printed in bold are significant ( $p < 0.05$ ).

Factor	Chisq	d.f.	$p$ -value
intercept	2.5200	1	0.1124
host source	2.3200	2	0.3135
<b>species and treatment</b>	<b>27.7275</b>	<b>3</b>	<b>&lt;0.0001</b>
host source $\times$ species and treatment	7.7015	6	0.2608



**Figure 3.2** A) Infection rates for *Mytilicola intestinalis* (blue) and *Mytilicola orientalis* (red) in single infections (squares connected by dashed lines) and co-infections (diamonds connected by solid lines). B) Host condition expressed as residuals of host dry weight corrected by mussel length, in which co-infections were only successful ones (i.e., mussels with one or more specimen of each parasite species present).

### Parasite length and reproduction

*Mytilicola orientalis* was larger than *M. intestinalis* after 80 days of infection in our experiment (Figure 3.3), but varied with species, treatment and host source (Table 3.3A, Figure 3.3). Parasite sex was also highly significant, as females are larger than males, and our models on female and male parasites only indicated that the difference between parasite lengths could be attributed to differences between female parasites (Table 3.3B, 3.3C). Our results further indicated that both parasite species grew at similar rates in single and co-infections (Tukey's HSD posthoc test on all parasites and females only shared the same results: 3 out of 4 between species comparisons were significant with  $p$ -values  $< 0.05$ , while both within species comparisons were not significant). In another GLMM testing parasite length as a factor of sex and the combined factor of host source, species and treatment, both sex and the combined factor were significant in analysis of deviance of the model (data not shown), while the Tukey's HSD posthoc test revealed that there were only significant differences between species or between species in combination with host sources, but never within species between treatments. Testing parasite length of only female parasites gave similar results, with length differing mainly between species, but never within species between treatments, while male parasites did not significantly differ in their



length between any of the factors. This further indicated that both parasite species grew at similar rates in single and co-infections.

In our experiment, only *M. orientalis* females carried egg sacs at the time of dissection after 80 days, whereas none of the *M. intestinalis* females in single (N = 39 mussels; N = 204 *M. intestinalis*) or co-infection (N = 39 mussels; N > 62 *M. intestinalis*) had egg sacs. In total, 22 female *M. orientalis* carried egg sacs, which were all found in Kiel mussels. In the single parasite treatment, three Kiel mussels carried 12 gravid *M. orientalis*. In the co-infection treatment, four Kiel mussels carried ten gravid *M. orientalis*. Therefore, out of 37 mussels experimentally infected with only *M. orientalis*, 12 parasites had egg sacs (0.324 egg sack pairs/mussel or 8.4% of *M. orientalis* females in the single infection treatment), and out of 39 co-infected mussels, 10 *M. orientalis* had egg sacs (0.256 egg sack pairs/mussel or 13.7% of *M. orientalis* females in the co-infection treatment, but note that this percentage likely is overestimated because of the removal of four mussels from the co-infection treatment whose parasites could not be determined to species reliably and

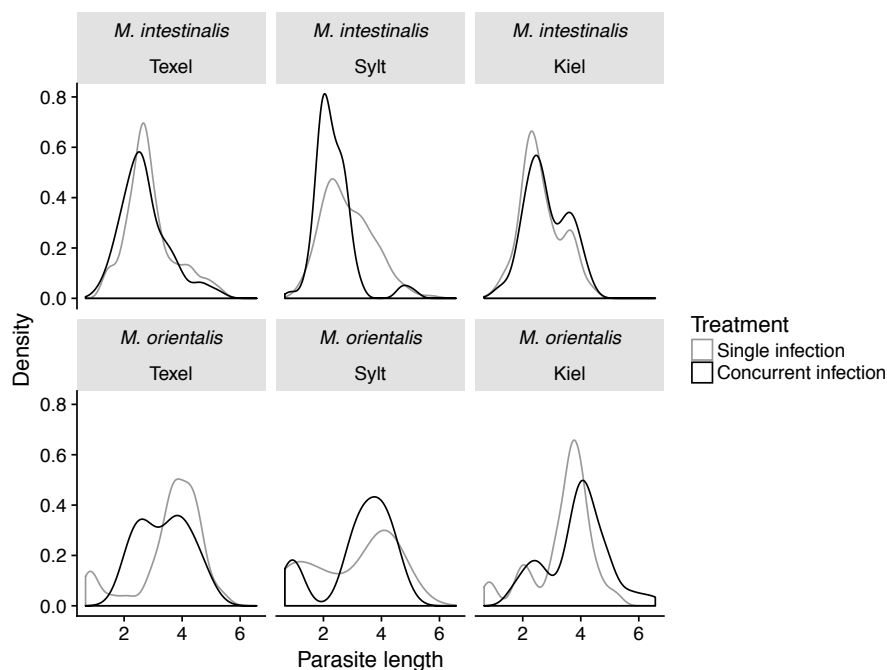
**Table 3.3** Analysis of deviance tables for the GLMM of parasite length (type III Wald chisquare test). The factors parasite species (levels: *M. intestinalis* and *M. orientalis*) and treatment (levels: single infections and co-infections) are combined. Factors printed in bold are significant ( $p < 0.05$ ).

Factor	Chisq	d.f.	p-value
A) All parasites, both sexes			
<b>intercept</b>	<b>838.3582</b>	<b>1</b>	<b>&lt;0.0001</b>
<b>sex</b>	<b>2367.5078</b>	<b>2</b>	<b>&lt;0.0001</b>
host source	5.0668	2	0.07939
<b>species and treatment</b>	<b>30.9359</b>	<b>3</b>	<b>&lt;0.0001</b>
<b>host source × species and treatment</b>	<b>12.7113</b>	<b>6</b>	<b>0.04786</b>
B) Only female parasites			
<b>intercept</b>	<b>502.1898</b>	<b>1</b>	<b>&lt;0.0001</b>
host source	1.9218	2	0.38254
<b>species and treatment</b>	<b>60.2765</b>	<b>3</b>	<b>&lt;0.0001</b>
<b>host source × species and treatment</b>	<b>13.9277</b>	<b>6</b>	<b>0.03045</b>
C) Only male parasites			
<b>intercept</b>	<b>362.1636</b>	<b>1</b>	<b>&lt;0.0001</b>
host source	2.7460	2	0.2533
species and treatment	4.0288	3	0.2584
host source × species and treatment	9.1774	5	0.1022

were left out of the parasite length analyses). Consequently, there was no significant difference in parasite reproduction between single and co-infections (ANOVA,  $\chi^2 = 0.0128$ , d.f. = 1,  $p = 0.9098$ ).

### Host condition

Host condition varied with host length and host source and the interaction between the two factors, but there was no overall difference in host condition between the control, infection and co-infection treatments (Table 3.4A). However, when looking within the single infection and co-infection treatments, also the host source  $\times$  parasite source interaction term was significant (Table 3.4B). The co-infections seemed to follow the *M. orientalis* pattern (Figure 3.2B). The individual models on dry weight per host source showed that only Sylt mussels infected with *M. intestinalis* had a significantly lower host condition, but none of the other infection combinations did (Table 3.4C–E, Table S3.1A–C).



**Figure 3.3** Density plots of parasite length for *M. intestinalis* and *M. orientalis* from Texel, Sylt and Kiel in single infections (grey) and co-infections (black). Note the two-peaked distribution of males and females.

**Table 3.4** Analysis of variance tables of optimal models of host condition of A) hosts from all sources (levels: Texel, Sylt, Kiel) in the different infection treatments (levels: non-infected or infected with *M. intestinalis*, *M. orientalis*, successfully co-infected; note that this factor was dropped in the optimal model); B) hosts from all sources infected with the different parasite sources (levels: *M. intestinalis*, *M. orientalis*, successfully co-infected); C–E) hosts from single sources (Texel C, Sylt D, Kiel E) infected with the different parasite sources (levels: *M. intestinalis*, *M. orientalis*, successfully co-infected). SS stands for sum of square and MS for mean square. Factors printed in bold are significant ( $p < 0.05$ ). See Table S3.1 for summary tables of the optimal GLMs of the single host sources.

Factor	d.f.	SS	MS	F-value	p-value
A) All hosts, all infection treatments					
<b>host length</b>	<b>1</b>	<b>2.6639</b>	<b>2.6639</b>	<b>18.818</b>	<b>&lt; 0.0001</b>
<b>infection intensity</b>	<b>1</b>	<b>1.5712</b>	<b>1.5712</b>	<b>11.098</b>	<b>0.00111</b>
<b>host source</b>	<b>2</b>	<b>17.2374</b>	<b>8.6187</b>	<b>60.881</b>	<b>&lt; 0.0001</b>
residuals	137	19.3946	0.1416		
B) All hosts, only parasite treatments					
<b>host length</b>	<b>1</b>	<b>2.3882</b>	<b>2.3882</b>	<b>18.1895</b>	<b>&lt; 0.0001</b>
<b>infection intensity</b>	<b>1</b>	<b>1.1593</b>	<b>1.1539</b>	<b>8.8297</b>	<b>0.00379</b>
<b>host source</b>	<b>2</b>	<b>12.7875</b>	<b>6.3938</b>	<b>48.6972</b>	<b>&lt; 0.0001</b>
parasite source	2	0.0066	0.0033	0.0253	0.975
<b>host source × parasite source</b>	<b>4</b>	<b>1.4775</b>	<b>0.3694</b>	<b>2.8133</b>	<b>0.0298</b>
<b>host length × host source</b>	<b>2</b>	<b>1.0607</b>	<b>0.5303</b>	<b>4.0393</b>	<b>0.0209</b>
residuals	91	11.9479	0.1313		
C) Texel hosts, only parasite treatments					
host length	1	0.09654	0.096543	1.1293	0.299
infection intensity	1	0.05713	0.057133	0.6683	0.422
parasite source	2	0.39787	0.198936	2.3270	0.120
residuals	24	2.05181	0.085492		
D) Sylt hosts, only parasite treatments					
<b>host length</b>	<b>1</b>	<b>3.2268</b>	<b>3.2268</b>	<b>32.2059</b>	<b>&lt; 0.0001</b>
<b>parasite source</b>	<b>2</b>	<b>1.1926</b>	<b>0.5963</b>	<b>5.9514</b>	<b>0.00666</b>
residuals	30	3.0058	0.1002		
E) Kiel hosts, only parasite treatments					
<b>host length</b>	<b>1</b>	<b>1.4491</b>	<b>1.44905</b>	<b>7.9368</b>	<b>0.00782</b>
infection intensity	1	0.0025	0.00250	0.0137	0.907
parasite source	2	0.0654	0.03270	0.1791	0.837
infection intensity × parasite source	2	0.7576	0.37880	2.0748	0.140
residuals	36	6.5727	0.18527		

## Discussion

Species occupying the identical habitat and using the same resources cannot coexist indefinitely in a limited environment and are therefore expected to compete (Gause 1935; Lotka 1932; Volterra 1926). By extension, concurrently infecting parasites can be expected to compete within their shared host – the mussel host is a finite resource (Feis *et al.* 2016) and *Mytilicola* parasites occupy the same habitat in the gut. Slightly different trophic enrichment levels were found for *M. intestinalis* (Gresty & Quarmby 1991) and *M. orientalis* (Goedknecht *et al.* 2017b), which could indicate that they have a different diet, although these studies from different locations may not be directly comparable. Examples of competition outcomes are for instance niche displacement, seen for example in co-infections of cestodes in stickleback guts (Chappell 1969), or the prevention of infection of the generalist on the specialist's host, observed for the two congeneric parasitic copepods, specialist *Lepeoptheirus thompsoni* and the generalist *L. europaensis* (Dawson *et al.*, 2000). Competitive exclusion, therefore, could also be expected between the two invasive *Mytilicola* species.

To our surprise neither a superiority effect, nor direct competition was found between specialist *Mytilicola intestinalis* and generalist *Mytilicola orientalis* within co-infections. Neither infection rates, nor parasite lengths of either parasite species in co-infections were different from single infections. A similar mean infection rate in single and in co-infections could mean that 1) there is no interference competition and 2) exploitation competition is not strong enough that it skews the infection rates or was reflected in growth processes. Indeed, differences in parasite length within species could only be attributed to sex and parasite species in combination with host source. It is possible that the overall competition experienced between the parasite species was as strong as the competition within species, for which we could not control in a substitution design experiment. Furthermore, while resources limit parasite maturation at high infection intensities of *M. intestinalis* infections (Feis *et al.* 2016), exploitation competition between *M. intestinalis* and *M. orientalis* did not seem to be stronger between than that within species. That is indicated by the lack of significant difference of parasite growth between *M. intestinalis* and *M. orientalis* in single compared to those in co-infections. Therefore, our results suggest that there is no direct competition (or facilitation) between *M. intestinalis* and *M. orientalis* and

that the higher infection success of *M. intestinalis* is more likely a result of specialist adaptation to the mussel host than of competitive superiority.

### **Invasion trajectory of *M. orientalis* and indirect forms of competition**

The relatively high prevalence of *M. orientalis* on Texel (Goedknecht et al. 2017) suggest that *M. orientalis* can successfully invade the niche of *M. intestinalis* and our recent field data suggests that this could also happen in the investigated mussel population. While we could not observe superiority in direct competition, the success of the *M. orientalis* invasion could also be driven by higher population growth rates. Time to maturation is a critical parameter determining population growth and the fact that we only observed gravid *M. orientalis* females might indicate that this species can reproduce faster. These females were, however, only observed in naïve Kiel hosts, but not in hosts from Texel and Sylt indicating that the evolved defence mechanisms of previously exposed hosts can prevent parasite growth and maturation. Moreover, also *M. intestinalis* from Texel showed a faster development than *M. intestinalis* from Sylt (M. Feis, unpublished data; Feis et al. 2016) and it is interesting to speculate whether the interaction with *M. orientalis* on Texel and its absence on Sylt might have selected for faster maturation, even though in terms of egg yolk transcription (Vitellogenin 2) parasites in Sylt mussels were upregulated, especially in Sylt parasites (significant interaction in *Chapter 5: Figures 5.3 and S5.1* and Feis et al. 2018). However, for both parasite species evidence of faster reproduction in the form of egg sacks was mainly seen in naïve hosts, suggesting that the co-evolved host defence modulates the development of the parasites in general.

The faster development of *M. orientalis* could result in a faster population growth and thereby explain the success of the new invader. This would also lead to asynchrony in the timing of reproduction of both species and priority effects could become important when faster reproduction opens up more infection windows. Timing is then what matters, and it is the interplay of eco-evolutionary dynamics involving infectivity of both parasites, resistance of the hosts, and reproduction success of both parasites that will determine population development.

Additionally, the different host use by the two congeners could lead to different degrees of population amplification, leading to a form of indirect competition. While previous experiments suggest that *M. intestinalis* can infect other host species (Hepper 1953, 1956), our experimental infection trials show that Pacific oysters as the

other dominant epibenthic filter feeder are not suitable hosts. Similarly, *M. gigas* seed was not readily infected by *M. intestinalis*, whereas seed of *O. edulis* and *M. edulis* was (Dare 1982). *Mytilicola orientalis*, on the other hand, also utilises other hosts and it is likely that *M. orientalis* populations are amplified by the wider host range within the Wadden Sea (Goedknecht *et al.* 2017a). The higher numbers of infective stages of *M. orientalis* produced within several different host species (Goedknecht *et al.* 2017a) in combination with faster development times could lead to faster population growth despite of lower infection rate in mussels. Host range and amplification would thus play a more important role than direct within-host competition in the invasion ecology of *M. intestinalis* and *M. orientalis*.

### **Effect of co-infections on the host**

In multiple infections, host damage inflicted by the parasites can be additive (i.e. the sum of both single effects), higher (multiplicative, Adedeji *et al.* 1989) or lower than single infections when the negative effects of the parasites cancel each other out (Massey *et al.*, 2004). Co-infections by both *Mytilicola* species in blue mussels did not lead to multiplicative effects on the host condition that were found in other model systems. For instance, mice hosting co-infections with nematode *H. polygyrus* and trypanosome *T. congolense* that were infected with both parasites simultaneously had highest mortality compared to control and sequentially infected mice, with the outcome of co-infections determined by the trypanosome, in particular anaemia and relative increase in spleen weight (Fakae *et al.* 1994). Interestingly, in the mice–trypanosome–nematode system, simultaneous or subsequent infection with trypanosomes did not influence worm establishment or fecundity (Fakae *et al.* 1994), which in part is a similar result as the *Mytilicola*–mussel simultaneous co-infections, for infection rate did not differ between single infections and co-infections, but egg production after 80 days did.

Similar to specialist *M. intestinalis* from Sylt causing lower body condition in their sympatric Sylt hosts, we also found a trend that Texel mussels infected with generalist *M. orientalis* from Texel tended to have a lower condition. This supports previous results, where mussels from Texel experimentally and naturally infected by sympatric *M. orientalis* also had a significantly reduced condition index (Goedknecht 2017). Extending infections to multiple combinations of hosts and parasites in our study showed that this effect is not general, but might depend on specific

combinations of host and parasites. Indeed we cannot rule out this confounding factor in our experimental design. However, if we only consider the condition of Sylt mussels, the co-infections should be expected to be in the middle, with the sympatric parasite influencing the host condition most, but they are not. Therefore multiple infections resembled pure *M. orientalis* infections (Figure 3.2B), although this trend was not statistically significant and an additional experiment would be needed to test this trend. We could already show that the lower body conditions in sympatric *M. intestinalis* infections could be linked to specific and concerted regulation of genes of the reactive oxygen species metabolism (Chapter 5, Feis *et al.* 2018) and a statistically significant correlation between body condition of pure *M. orientalis* and co-infections would indicate that the immune reaction of mussels was dominated by *M. orientalis*. It would be interesting to follow up on the currently described experiment with similar methods and analyses. We further speculate that the recent appearance of *M. orientalis* could not have left sufficient time to evolve such a specific response to this new invader and might therefore explain the potential immune dominance of this species.

### **Future scenario for uninvaded regions**

Our results also allow some projections for the ongoing invasion of *M. orientalis* on Sylt. The lower infection success and the absence of direct competition suggests that *M. orientalis* might not invade the oyssel reefs on Sylt easily when solely relying on mussels as host, because the prevalence and infection intensity of *M. intestinalis* is already very high here (Elsner *et al.* 2010; Goedknecht *et al.* 2017a). Based on low suitability of oysters as hosts for *M. intestinalis*, oysters are free habitat for *M. orientalis* only, giving it the opportunity to invade by colonizing oysters first, then spilling over to mussels. This is also reflected in the field data from Sylt, as by 2016 13% of the oysters and 2% of the mussels were already infected with *M. orientalis*. However, a priority effect could also explain the observation, since most mussels are occupied by *M. intestinalis*, which makes subsequent infections by *M. orientalis* less likely. Our prediction is that a priority effects may be of importance for specialist *M. intestinalis*, as it only infects blue mussels, but that it may be of lesser importance for generalist *M. orientalis*, that may infect other host species instead. It was already shown that presence or absence of a previous *M. intestinalis* infestation did not affect

the subsequent infection success of *M. intestinalis* copepodites (Gee & Davey 1986a), but a test between *M. intestinalis* and *M. orientalis* has not been performed yet.

Therefore, to reflect natural infestations and concurrent infestations by *Mytilicola* spp. better, further research could focus on testing for priority effects by performing sequential infection experiments to give one species a head start. In our experiment both parasite species had an equal chance at conquering space within the host and we did not find a superiority effect. Such a superiority effect was shown in a cross-infection experiment with specialist and generalist canestriniid mites on two species of lucanid beetles, and the superiority of the specialist persisted regardless of which species infected first (Okabe *et al.* 2012). As in our study, no superiority was found between the two microsporidians *Nosema apis* and *Nosema ceranae* co-infecting honeybees *Apis mellifer* (Natsopoulou *et al.* 2015). There, however, an asymmetrical priority effect was found with the invader *N. ceranae* inhibiting *N. apis* more than vice versa, which favoured the spread of the invader (Natsopoulou *et al.* 2015). Thus a sequential infection experiment of *M. intestinalis* versus *M. orientalis* within mussels may thus yield more precise insights into what might happen during the ongoing *M. orientalis* invasion the oysel reefs in the Wadden Sea, and in addition, in the other regions where both hosts now co-occur, but the parasites have not invaded yet (e.g., Norway, Hopkins 2001; Mortensen *et al.* 2015).

## Conclusion

We conclude that there is no superiority between the two invasive parasitic copepods *Mytilicola intestinalis* (a blue mussel specialist in the Wadden Sea) and *Mytilicola orientalis* (a generalist infecting amongst others blue mussels and Pacific oysters). Hence, superiority could not have played a role in the invasion success of the newer invader *M. orientalis* in previously invaded regions (Goedknecht, 2017). In fact, the lower infection rates of *M. orientalis* might indicate that *M. orientalis* pays the price of generalism by being inferior to *M. intestinalis* and that its potential immunodominance might play a role here, but further experiments are needed to test this. Furthermore, since *M. intestinalis* does not readily infect Pacific oysters, Pacific oysters can act as an amplifier for *M. orientalis* or may even act as a diluter for *M. intestinalis* when they enter this unsuitable host (Thieltges *et al.* 2009; Welsh *et al.*



2014). These findings back up the work by Goedknecht *et al.* (2017a), who underline the importance of spillover of *M. orientalis* from Pacific oysters and other hosts in the complex ecological interactions between native blue mussels, invasive Pacific oysters and the two *Mytilicola* parasites. Ongoing parasite invasions thus offer the opportunity to study how interspecific competition influences host–parasite co-evolution in real time.

### **Acknowledgements**

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## Supplementary materials

**Table S3.1** Summary of the optimal dry weight models of A) Texel hosts, B) Sylt hosts, C) Kiel hosts. ANOVA of these models are described in Table 3.5 C, D and E. Factors printed in bold are significant ( $p < 0.05$ ).

	estimate	std. error	<i>t</i> value	Pr(>  <i>t</i>  )
A) Texel hosts only				
<b>intercept</b>	<b>-1.77222</b>	<b>0.65237</b>	<b>-2.717</b>	<b>0.0120</b>
host length	0.02344	0.01565	1.498	0.1473
infection intensity	-0.01937	0.01131	-1.712	0.0998
parasite source <sub>co-infection</sub>	-0.25789	0.15336	-1.682	0.1056
parasite source <sub><i>M. orientalis</i></sub>	-0.29485	0.14793	-1.993	0.0577
B) Sylt hosts only				
<b>intercept</b>	<b>-6.02352</b>	<b>0.65851</b>	<b>-9.147</b>	<b>&lt; 0.0001</b>
<b>host length</b>	<b>0.09125</b>	<b>0.01516</b>	<b>6.018</b>	<b>&lt; 0.0001</b>
<b>parasite source<sub>co-infection</sub></b>	<b>0.34947</b>	<b>0.13315</b>	<b>2.625</b>	<b>0.0135</b>
<b>parasite source<sub><i>M. orientalis</i></sub></b>	<b>0.41681</b>	<b>0.13153</b>	<b>3.169</b>	<b>0.00351</b>
C) Kiel hosts only				
<b>intercept</b>	<b>-4.45047</b>	<b>0.86322</b>	<b>-5.156</b>	<b>&lt; 0.0001</b>
<b>host length</b>	<b>0.05566</b>	<b>0.01817</b>	<b>3.064</b>	<b>0.00412</b>
infection intensity	0.01806	0.02686	0.672	0.506
parasite source <sub>co-infection</sub>	-0.29577	0.51955	-0.569	0.573
parasite source <sub><i>M. orientalis</i></sub>	0.44787	0.42973	1.042	0.304
infection intensity × parasite source <sub>co-infection</sub>	0.01776	0.03892	0.456	0.651
infection intensity × parasite source <sub><i>M. orientalis</i></sub>	-0.04994	0.03499	-1.427	0.162





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Biological invasions and host–parasite co-evolution:  
different co-evolutionary trajectories along separate  
parasite invasion fronts

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## Abstract

Host–parasite co-evolution has rarely been observed in natural systems. Its study often relies on microparasitic infections introducing a potential bias in the estimation of the evolutionary change of host and parasite traits. Using biological invasions as a tool to study host–parasite co-evolution in nature can overcome these biases. We demonstrate this with a cross-infection experiment in the invasive macroparasite *Mytilicola intestinalis* and its bivalve host, the blue mussel *Mytilus edulis*. The invasion history of the parasite is well known for the southeastern North Sea and is characterised by two separate invasion fronts that reached opposite ends of the Wadden Sea (i.e. Texel, The Netherlands and Sylt, Germany) in a similar time frame. The species' natural history thus makes this invasion an ideal natural experiment to study host–parasite co-evolution in nature. We infected hosts from Texel, Sylt and Kiel (Baltic Sea, where the parasite is absent) with parasites from Texel and Sylt, to form sympatric, allopatric and naïve infestation combinations, respectively. We measured infection rate, host condition and parasite growth to show that sympatric host–parasite combinations diverged in terms of pre- and post-infection traits within <100 generations since their introduction. Texel parasites were more infective and more efficient at exploiting the host's resources. Hosts on Texel, on the other hand, evolved resistance to infection, whereas hosts on Sylt may have evolved tolerance. This illustrates that different co-evolutionary trajectories can evolve along separate invasion fronts of the parasite, highlighting the use of biological invasions in studies of host–parasite co-evolution in nature.

*Keywords:* host–parasite interactions; infectivity/resistance; virulence/tolerance; local adaptation; parasitic copepod

## Introduction

Theoretical predictions of the dynamics underlying host–parasite co-evolution (Hamilton *et al.* 1990; Sasaki 2000; Van Valen 1973) have been confirmed in study systems that employ experimental evolution (Berenos *et al.* 2011a; Gomez & Buckling 2011; Joop & Vilcinskas 2016; Schulte *et al.* 2010). However, evidence from natural systems supporting these proof-of-principle observations is limited, although some natural systems (e.g., the water flea *Daphnia magna* and its castrating bacterial parasite *Pasteuria ramosa*) offer the opportunity to cross-infect different generations of hosts and parasites sampled directly from the environment, confirming that negative frequency-dependent selection can also act in natural populations (Decaestecker *et al.* 2007).

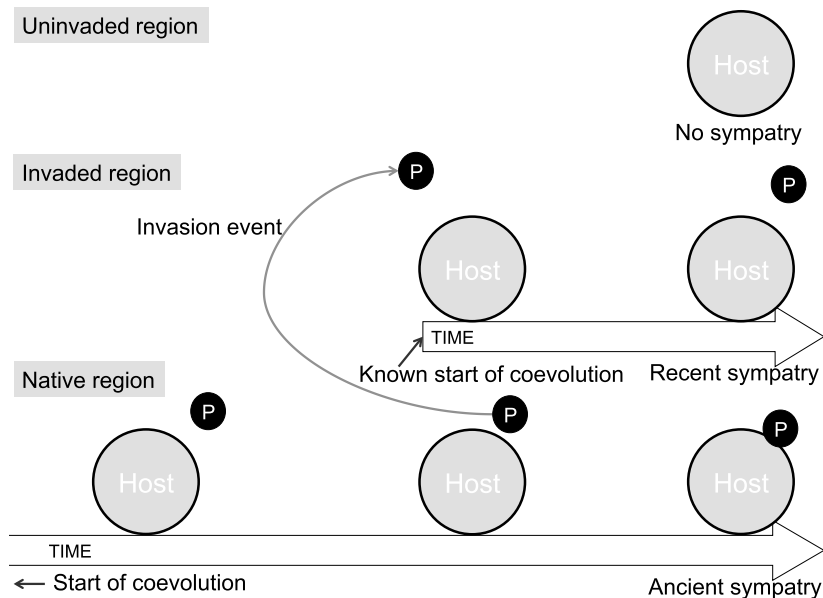
The above-mentioned studies use hosts with short generation times that are infected by microparasites. It is likely to observe co-evolution in these systems, because often microparasites tend to be virulent, favouring host responses. Furthermore, short host generation times facilitate evolutionary responses. In contrast to host-microparasite interactions, host-macroparasite combinations have been investigated far less often and experimental studies are scarce (but see the study on *Potamopyrgus antipodarum* and its castrating trematode *Microphallus* sp.; Dybdahl & Lively 1998; Koskella & Lively 2007, 2009). More support for host-macroparasite interactions comes from local adaptation experiments (reviewed in Greischar & Koskella 2007; Hoeksema & Forde 2008; Kaltz & Shykoff 1998) that represent proxies for co-evolution, but often fail to capture the temporal dimension of co-evolution.

It thus becomes clear that our empirical understanding of host–parasite co-evolution is based on phylogenetic and experimental biases that fail to encompass the phylogenetic variety of host–parasite interactions, as well as the variety of their underlying dynamics that are present in nature.

Biological invasions can overcome several of these biases and therefore represent excellent opportunities to study the ecological and evolutionary effects of parasites and pathogens (Goedknecht *et al.* 2016) and thus, co-evolutionary processes. Invasions are natural experiments with phylogenetically diverse combinations of hosts and parasites (Goedknecht *et al.* 2016). Additionally, the time frame of evolutionary changes is often known since it coincides with the time of invasion. It is therefore

possible to estimate a rate of change by comparing native, invasive and naïve combinations of hosts and parasites after the invasion event (Figure 4.1). In this way, biological invasions can add a time frame to local adaptation experiments. Finally, several invasion scenarios in which only the host or the parasite invade, or in which both host and parasite co-invade, lead to different predictions regarding the underlying evolutionary dynamics (reviewed in Goedknecht *et al.* 2016).

The invasive parasite *Mytilicola intestinalis* (Copepoda: Cyclopoida) offers a compelling natural history background to test several predictions of host–parasite co-evolution in the wild. Originating from the Mediterranean Sea (Steuer 1902, 1905) where it infests the Mediterranean mussel *Mytilus galloprovincialis*, the parasite invaded the North Sea and spread southwest and north in two fronts (see Figure 4.2). The direct life cycle of this parasite limits co-evolution to one principal host, the blue mussel *Mytilus edulis*, in its invaded range, and controlled infections (Gee & Davey 1986a; Hepper 1953) can be applied to previously treated mussels (Blateau *et al.* 1992) to generate experimental combinations of host and parasite populations. Since *M. intestinalis* creates lesions in the epithelium of the intestinal walls of its host,

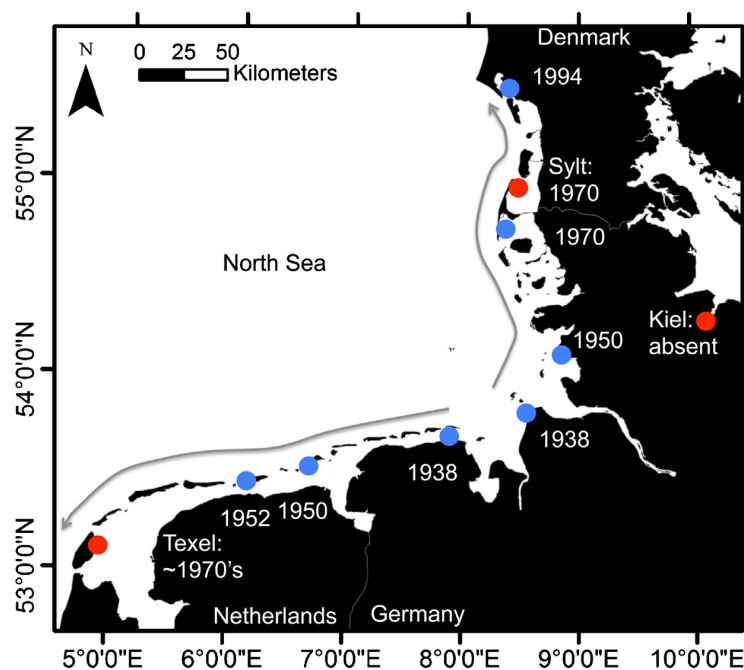


**Figure 4.1** Co-evolution in biological parasite invasions. While in native regions host–parasite co-evolution occurred over long and usually unknown timespans (ancient sympatry), invasions of parasites clearly define the onset of new co-evolutionary interactions with new hosts (recent sympatry). In the uninvaded region there is no sympatry with naïve hosts. “P” symbolises the parasite and the distance of the parasite to the host represents the co-evolutionary relation between host and parasite. Reciprocal infection experiments with hosts and parasites of different levels of sympatry, covering a range from ancient sympatry over recent sympatry to no sympatry, can thus be used to estimate the rate of evolutionary change.



especially at higher infection intensities (Couteaux-Bargeton 1953; Watermann *et al.* 2008), and has been associated with mass mortalities (Blateau *et al.* 1992; Korringa 1950; Meyer & Mann 1950), selection for host resistance seems likely.

Pre- and post-infection traits of hosts and parasites can be separated within the mussel–*Mytilicola* system. The pre-infection traits are parasite infectivity, which is the ability to infect the host, and host resistance, i.e. the host’s ability to prevent infections. Once infected, co-evolution can occur for post-infection traits, i.e. host tolerance, which is the capability of the host to deal with infection, and parasite virulence, which is the harm inflicted on the host that should correlate with the ability to exploit the host. Both pre- and post-infection traits of hosts and parasites are tightly coupled and are therefore difficult to disentangle. While the proportion of successful infections resulting from exposure to a defined number of infective stages is a precise estimator of infectivity and resistance, tolerance and virulence can only be derived indirectly from host body condition in relation to parasite load. Nevertheless, pre- and post-infection traits can be separated in this system, offering the opportunity to



**Figure 4.2** Map of the invasion route of *Mytilicola intestinalis* in the Wadden Sea, a long stretch of intertidal areas and barrier islands in the southeastern North Sea. *M. intestinalis* was first found in 1938 near Wilhelmshaven and Cuxhaven (Caspers 1939). The parasite spread north and southwest towards both ends of the Wadden Sea (Meyer & Mann 1950) and reached Germany’s most northern Wadden island, Sylt, by 1970 (Dethlefsen 1972). Although *M. intestinalis* was still absent west of the Dutch Wadden island Ameland in the year 1968 (Korringa 1968), it probably arrived on Texel during the 1970s.

investigate the evolutionary trajectories involving these traits.

Here, we describe these co-evolutionary trajectories for the host *M. edulis* and the parasite *M. intestinalis* after its invasion in relation to naïve hosts lacking co-evolutionary interactions. In particular, we answer how invasive parasites affect naïve hosts, and if similar host–parasite interaction patterns were found at the two different invasion fronts in the Wadden Sea.

## **Materials and methods**

### **Field collection of mussels and treatment against previous infestations**

Mussels in the size category of 3.5–5.0 cm shell length were collected from mixed mussel and oyster beds at the tidal flats of Vlakte van Kerken on the island of Texel (53°09'17.7"N, 04°53'36.1"E) and on the island of Sylt, Germany (55°02'17"N, 08°26'32"E) in June/July 2014 (Figure 2). Naïve hosts were collected from a mussel bed in Kiel Harbour, Germany (Baltic Sea, 54°19'48.0"N, 10°09'00.0"E) and were adapted over a two-week period to the higher salinity of the North Sea. After transfer to the laboratory, mussels were kept in a climate-controlled room at 18 °C in an aquarium system with semi-continuous flow-through of fine-filtered seawater (30 µm) that was continuously re-filtered (50 µm) and UV-treated to prevent cross-infection throughout the experimental phase.

To eliminate previous infestations with *Mytilicola* spp. before the start of the experiment, all mussels were treated with Dichlorvos Pestanal (DDVP), following Blateau *et al.* (1992). Per bath, 100 mussels were put into 1 l of seawater with 30 mg DDVP for 4 h. After a recovery period of at least two weeks in aerated, filtered and UV radiation-treated seawater that was changed at least once per day, mussels were transferred again into a 1 l bath with 30 mg DDVP for 2 h. The experiment was initiated after a second recovery period of at least three weeks under the same protocol. At the start of the experiment, all mussels could retract their mantle fringes and close their shells completely, indicating that they had recovered from the DDVP treatment.

### **Experimental mussel infection**

Egg sacks were obtained from individual gravid female *M. intestinalis* and were put into 24-well plates with ultra-filtrated seawater (0.45 µm). Eggs hatched within 1–12 days. Since the free-swimming larval stages are short-lived and only the first copepodite stage is infective to mussels (Hockley 1951; Pesta 1907), we infected individual mussels by pipetting 24 swimming copepodites into a 200 ml Kautex bottle containing a single mussel in ultra-filtrated seawater. Copepodites were taken from one mother or were a mix from two mothers. After 24h the water was filtered through a 50 µm mesh sieve, which was checked under the stereomicroscope for remaining copepodites that were counted and removed. The bottle with the mussel was randomly placed into the aquarium system providing individual flow-through for each bottle. We infected mussels from Texel, Sylt and Kiel with parasites from Texel (each group, n = 7) and parasites from Sylt (each group, n = 15) and also included uninfected controls (n = 15).

Mussels were fed three times per week with  $3.9 \times 10^7$  cells of *Isochrysis* 1800TM (0.01 ml/mussel; CCMP 1324 T.ISO; Reed Mariculture, Campbell, CA, USA). During feeding, flow-through was interrupted for 3 h. Mortality was noted after each feeding event. Bottles were repositioned randomly twice per week.

After 80 days, mussels were dissected under a dissection microscope (6.3×). All parasites were removed from the gut and intestine. We measured host length to the nearest 0.01 mm with a digital calliper and flesh dry weight to the nearest 0.0001g on a precision balance. Mussel flesh was separated from the shell and dried in an oven at 50 °C until constant weight. Parasites were sexed, counted and photographed under a dissection microscope for determining the length from head to tail (Leica QWIN imaging software; Leica Microsystems Imaging Solutions, Ltd., Cambridge, UK).

### **Data handling and statistical analyses**

All analyses were done in the R statistical environment version 0.99.893 (R Core Team, 2015). To optimise models, we used a stepwise selection procedure based on the Akaike information criterion (AIC). Results from the optimal models were not qualitatively different from those of the full models.

Host survival was tested with a binomial generalised linear model (GLM) estimating the effects of treatment (control or infected), host source and parasite

source plus the interactions of treatment  $\times$  host source and host source  $\times$  parasite source.

Infection rate was used as a measure of infectivity of the parasite and resistance of the host. Infection rates were calculated by dividing the infection intensity by the exposure dose (number of parasites that infected the host, calculated as 24 minus the number of larvae that were still present in the water after 24 h, which were removed). We fitted optimal binomial GLMs testing successful against failed infections as a function of host and parasite sources plus the interaction term. We first calculated a model for all exposed mussels, and subsequently excluded Kiel hosts to separate recent sympatry combinations from naïve hosts.

Dry weight of the mussel flesh was used as a measure for the host condition, representing the relationship of host tolerance and parasite virulence. We defined parasite virulence as the reduction in host condition due to infection and host tolerance as the ability to deal with infection by parasites without suffering from condition loss, i.e. reduced slope of body condition as a function of infection intensity. Dry weight data were normally distributed after applying a log transformation (Shapiro-Wilk normality test,  $p = 0.5573$ ). Because weight increases with body size, we corrected for host length by fitting it as the first term in the model. One mussel from Kiel infected with Sylt parasites was excluded from the dataset because the dry weight was more than 3 standard deviations away from the mean dry weight of that group (mean = 0.2237 g; sd = 0.2035; outlier = 0.9165 g), which was most likely due to a measurement error. Our sequential model fitting strategy for host condition was as follows. First, we tested for a treatment effect by optimising the full linear model using host dry weight (log-transformed) as a function of host length, host source, the treatment (control or infected) plus the interactions between all main effects. Second, to test for the effect of parasite source, we modelled log-transformed dry weight in a linear model as a function of host length, infection intensity, host source, parasite source and the interactions between infection intensity and host source, and host source and parasite source (terms in the full model). Finally, we fitted the same dry weight model with only Texel and Sylt hosts to separate recent sympatry combinations from naïve hosts.

We used mean parasite length and parasite development rate, expressed as the proportion of adults at time of dissection, as components of parasite fitness. Mean parasite length per host was taken as a measure for parasite growth and was normally

distributed (Shapiro-Wilk normality test,  $p = 0.508$ ). Mean parasite length was tested with a GLM as a function of infection intensity, sex ratio, host dry weight, parasite source and host source plus the two-way and three-way interaction terms between host dry weight, host source and parasite source (terms in the full model). We included host dry weight in the model to test if there is a dependency of the parasite growth rate on the availability of host resources. The slope of the parasite mean length by host dry weight relationship indicates exploitation efficiency. Parasite development rate was tested with a binomial GLM as a function of infection intensity, host source, parasite source and all interactions (full model). This test was also repeated for the dataset without naïve Kiel hosts.

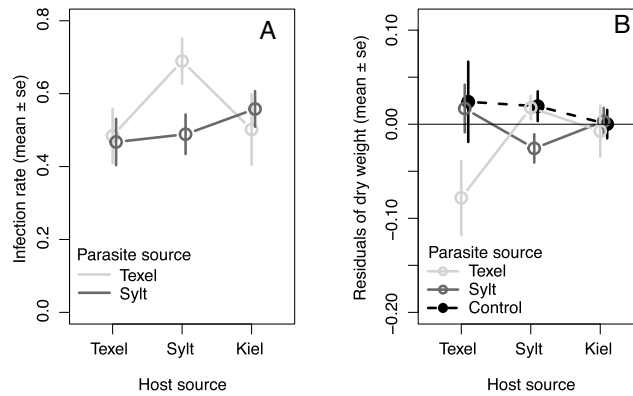
## Results

All mussels that were not exposed to parasites were free of infections at the end of the experiment, demonstrating that all found infections resulted from experimental exposure and not from any cross-infections between individual hosts or residual infections from the field. All parasites from the experimental infections were identified as *M. intestinalis* based on morphology (Steuer 1905), showing that we successfully selected egg sacks from gravid *M. intestinalis* females only. Hosts in Kiel were indeed naïve, as no parasites were found in 190 mussels dissected before the experiment and as none were found in the baths after DDVP treatment. Sixteen mussels died throughout the experiment. Even though mortality was higher in the recent sympatry host sources (18.9% mortality in mussels from Texel and 16.2% in mussels from Sylt) than in the naïve host source (8.3% mortality in mussels from Kiel), we found no significant difference in host survival between host sources ( $Deviance_{\text{host source}} = 1.97$ , d.f. = 2,  $p = 0.37$ ), between parasite sources ( $Deviance_{\text{parasite source}} = 0.046$ , d.f. = 1,  $p = 0.83$ ), between treatments ( $Deviance_{\text{treatment}} = 1.79$ , d.f. = 1,  $p = 0.18$ ) nor in the interaction between host and parasite sources ( $Deviance_{\text{host source} \times \text{parasite source}} = 3.99$ , d.f. = 2,  $p = 0.14$ ).

### Infectivity and resistance

Infection rates in relation to observed exposure dose ranged from 0 to 0.958 with a mean ( $\pm$  SE) of  $0.526 \pm 0.0265$ . In our experiment, parasites from Texel were on

average more infective than parasites from Sylt (mean infection rate  $\pm$  SE =  $0.565 \pm 0.0479$  and  $0.507 \pm 0.0317$ , respectively,  $Deviance_{\text{parasite source}} = 4.46$ , d.f. = 1,  $p = 0.035$ ; Figure 4.3A) owing to the fact that the infection rate of Texel parasites was significantly higher in mussels from Sylt than from Texel and Kiel ( $Deviance_{\text{host source}} = 7.77$ , d.f. = 2,  $p = 0.021$ ;  $Deviance_{\text{parasite source}} = 4.46$ , d.f. = 1,  $p = 0.035$ ;  $Deviance_{\text{host source} \times \text{parasite source}} = 15.40$ , d.f. = 2,  $p < 0.001$ ; total d.f. = 57; Figure 4.3A). The infection rate of Sylt parasites did not show a significant difference between Texel and Sylt hosts (post-hoc  $\chi^2_{\text{d.f.} = 1, 23} = 0.384$ ,  $p = 0.54$ ) or between Sylt and Kiel hosts (post-hoc  $\chi^2_{\text{d.f.} = 1, 25} = 2.973$ ,  $p = 0.085$ ).



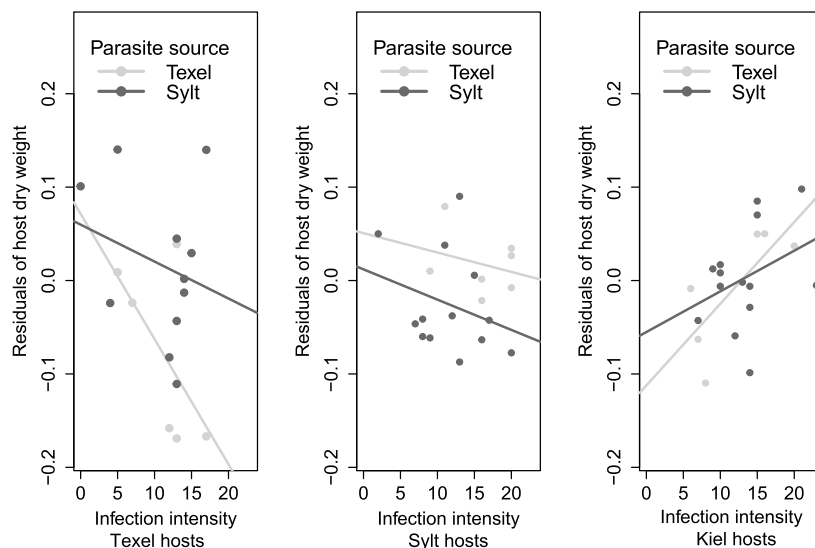
**Figure 4.3** Pre- and post-infection traits of host and parasite. **(A)** Mean parasite infection rate per host ( $\pm$  SE), representing host resistance vs. parasite infectivity. **(B)** Mean host dry weight (controlled for host length) as a measure of body condition ( $\pm$  SE), representing host tolerance vs. parasite virulence.

**Table 4.1** Analysis of variance (ANOVA) of optimal models of the host condition (log-transformed host dry weight). Significant  $p$ -values are printed in bold.

Factor	d.f.	sum of squares	mean square	$F$ -value	$p$ -value
<b>(A) Infected vs. non-infected hosts</b>					
Host length	1	1.3399	1.3399	9.7226	<b>0.0025</b>
Host source	2	13.2598	6.6299	48.1076	<b>&lt;0.0001</b>
Treatment	1	0.1540	0.1540	1.1175	0.2934
Host length $\times$ treatment	2	0.5134	0.5134	3.7254	0.0568
Residuals	87	11.9898	0.1378		
<b>(B) Infected hosts only</b>					
Host length	1	0.8693	0.8693	7.1700	<b>0.0101</b>
Infection intensity	1	0.2036	0.2036	1.6792	0.2014
Host source	2	8.8141	4.4071	36.3476	<b>&lt;0.0001</b>
Parasite source	1	0.0051	0.0051	0.0419	0.8388
Infection intensity $\times$ host source	2	0.7885	0.3943	3.2518	<b>0.0476</b>
Host source $\times$ parasite source	2	1.1566	0.5783	4.7696	<b>0.0130</b>
Residuals	47	5.6987	0.1212		
<b>(C) Full reciprocal cross-infected combinations only (no naïve Kiel hosts)</b>					
Host length	1	0.2095	0.2095	2.3673	0.1340
Infection intensity	1	0.8354	0.8354	9.4386	<b>0.0044</b>
Host source	1	6.8258	6.8258	77.1229	<b>&lt;0.0001</b>
Parasite source	1	0.0662	0.0662	0.7482	0.3937
Host source $\times$ parasite source	1	1.2666	1.2666	14.3108	<b>&lt;0.001</b>
Residuals	31	2.7437	0.0885		

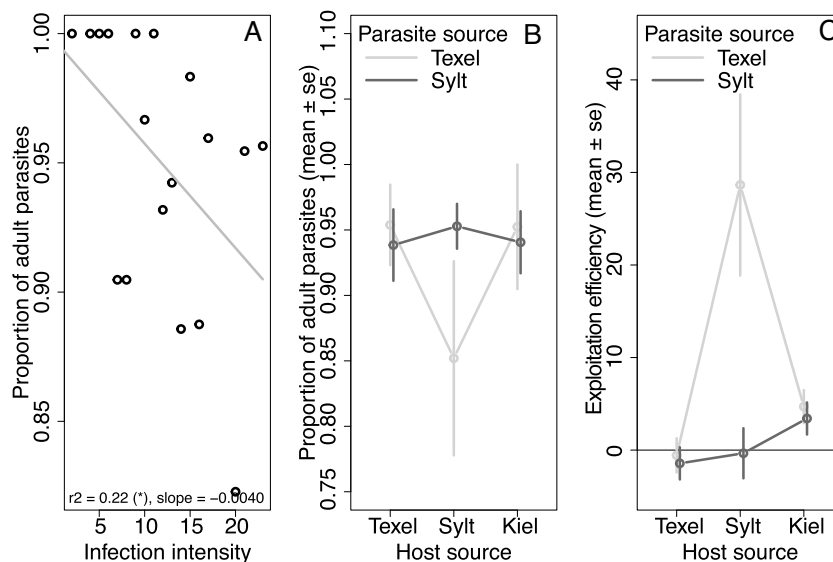
## Virulence and tolerance

While hosts from different sources had significantly different dry weights, we could not find a significant difference in host condition between uninfected and infected hosts (treatment factor in Table 4.1A; Figure 4.3B), indicating that infection did not lead to lower conditions in general. This effect could, however, mainly be attributed to the inclusion of naïve hosts into the analyses. Leaving naïve Kiel mussels out of the analyses, we observed that in sympatric combinations (Texel hosts with Texel parasites; Sylt hosts with Sylt parasites), condition was significantly lower than in the allopatric combinations (post-hoc tests, Texel:  $\chi^2_{d.f. = 1, 11} = 0.474$ ,  $p = 0.031$ , Sylt:  $\chi^2_{d.f. = 1, 22} = 6.872$ ,  $p < 0.0001$ ) that did not differ significantly from the control animals (Table 4.1C and Figure 4.3B). On Texel, sympatric parasites reduced the mean host dry weight by 28.21% and on Sylt, by 18.75%. Our model selection strategy only included length as a significant factor in the analyses with naïve hosts from Kiel. In the analyses only containing the reciprocal pairs of hosts and parasites (i.e. Texel and Sylt), host length was not significantly different. Infection intensity and the host  $\times$  parasite interaction term explained a significantly larger proportion of the variation of host dry weight than in the model containing the naïve hosts. In naïve hosts, the parasite groups did not differ significantly (Table 4.1B and C). Although the slope of the sympatric combination on Texel was considerably steeper, we could not detect a significant three-way interaction term in the host dry weight models (Table 4.1B and C and Figure 4.4).



**Figure 4.4** Tolerance relationships between hosts and parasites, measured as host dry weight (corrected for host length) over infection intensity.

Parasite development rate was negatively correlated with infection intensity (Figure 4.5A). This effect was particularly strong for Sylt hosts infected with Texel parasites, reflecting the higher infection rate in the Sylt hosts by Texel parasites (Table 4.2A and Figures 4.3A and 4.5B). Host source was not a significant factor in the model when naïve hosts were excluded (Table 4.2B). Parasite length differed significantly between the sexes; females were on average 47.8% larger than male parasites (GLMM, d.f. = 2,  $F$ -value = 614.37,  $p < 0.0001$ ). Mean parasite length per host varied depending on the sex ratio and on the interaction term between host dry weight, parasite source and host source in the model with all host sources (Table 4.2C), but varied depending on the sex ratio and the combination of host source  $\times$  parasite source in the model without naïve hosts (Table 4.2D). Mean parasite length did not depend on the infection intensity (Table 4.2C and D). The slope of parasite mean length depending on host dry weight for the different host–parasite combinations represents the exploitation efficiency of the parasite (Figure 4.5C). Parasites from both sources efficiently exploited naïve mussels from Kiel; however, in recent sympatry combinations, only Texel parasites were able to strongly exploit resources of Sylt hosts (Figure 4.5C).



**Figure 4.5** Parasite development and exploitation efficiency. **(A)** Parasite development rate given as the proportion of adults as a function of infection intensity of all locations together. **(B)** Parasite development and **(C)** slope of the parasite mean length by host dry weight relationship, i.e. exploitation efficiency of the host's resources by the parasite. Positive values indicate a higher availability of resources that can be more efficiently exploited, while values around zero indicate that parasite mean length is independent of host dry weight and negative values would point out that more host resources are available for the host itself, for, e.g., immune response.



**Table 4.2** Analysis of deviance of the optimal models of parasite development (**A** and **B**) and parasite length (**C** and **D**). Significant *p*-values are printed in bold.

Factor	d.f.	Deviance	Residual d.f.	Residual deviance	<i>p</i> -value
<b>(A) Parasite development</b>					
Null			54	104.410	
Infection intensity	1	7.1382	53	97.272	<b>0.0075</b>
Host source	2	7.1757	51	90.096	<b>0.0277</b>
Infection intensity × host source	2	15.6060	49	74.490	<b>&lt; 0.001</b>
<b>(B) Parasite development without naïve Kiel mussels</b>					
Null			34	72.191	
Infection intensity	1	22.18	33	50.011	<b>&lt; 0.0001</b>
<b>(C) Parasite length</b>					
Null			54	16.3575	
Sex ratio	1	6.2796	53	10.0780	<b>&lt; 0.0001</b>
Host dry weight	1	0.4704	52	9.6075	0.0841
Parasite source	1	0.0762	51	9.5313	0.4870
Host source	2	0.5358	49	8.9956	0.1829
Host dry weight × parasite source	1	0.0797	48	8.9159	0.4772
Host dry weight × host source	2	0.3625	46	8.5535	0.3168
Parasite source × host source	2	0.8874	44	7.6661	0.0600
Host dry weight × parasite source × host source	2	1.0436	42	6.6224	<b>0.0365</b>
<b>(D) Parasite length without naïve Kiel mussels</b>					
Null			34	11.5952	
Infection intensity	1	0.0316	33	11.5636	0.6691
Sex ratio	1	5.7242	32	5.8393	<b>&lt; 0.0001</b>
Host dry weight	1	0.0241	31	5.8153	0.7091
Parasite source	1	0.0612	30	5.7541	0.5521
Host source	1	0.0132	29	5.7409	0.7827
Host dry weight × parasite source	1	0.0013	28	5.7396	0.9301
Host dry weight × host source	1	0.1338	27	5.6058	0.3791
Parasite source × host source	1	0.9886	26	4.6172	<b>0.0168</b>
Host dry weight × parasite source × host source	1	0.2934	25	4.3238	0.1928

## Discussion

Our results show that parasites from the Wadden Sea were able to exploit naïve hosts from Kiel but did not show any difference in pre-infection and post-infection traits. In recent sympatry combinations, however, coupled pre-infection traits (infectivity and resistance) and post-infection traits of parasites and hosts (virulence and tolerance) evolved differently along the separate invasion fronts of *M. intestinalis* in the Wadden Sea. Since it is hard to disentangle these coupled trait combinations, it is difficult to conclude whether hosts or parasites evolved at higher rates. Using a naïve host population can partly circumvent this problem, because a directed evolutionary response of the host can be ruled out in the absence of the parasite.

### **Naïve vs. recent sympatry hosts**

If mainly hosts evolved resistance or tolerance to the parasite infection we would expect that Kiel hosts, which do not share an evolutionary history with the parasite, would have higher infection rates and lower body conditions than coevolved hosts. However, Kiel hosts did not show a decrease in dry weight due to parasite infection, as was observed for sympatric infections in Texel and Sylt hosts, nor differences in susceptibility between parasite sources. As these parasites do not have any co-evolutionary history with the Baltic hosts, they are not adapted to infect these hosts, and the host does not have a coevolved immune response that is specific to these parasites. Infection intensity and the host source  $\times$  parasite source interaction term explained a larger proportion of the variation of host dry weight in the model without the naïve hosts, further supporting that co-evolution could only be observed in the two localities where infections occur naturally, but not in the naïve population. Whereas the Sylt and Texel hosts seem to have a specific response to fight off sympatric infections, Kiel mussels did not show this response. Parasites from both sources were equally efficient in exploiting the naïve host, but their exploitation efficiency was reduced in sympatric combinations (Figure 4.5C), further supporting the notion that co-evolution is a local process.

The lack of specific patterns of all parasite traits in naïve hosts seems to suggest a lack of specific parasite adaptation. Mussel populations in the Baltic Sea are more distantly related to populations in the North Sea than populations within the North Sea are to each other, and the Baltic populations represent hybrids containing only 80% of the *M. edulis* genome (Stuckas *et al.* 2009). This strong differentiation might have masked any specific adaptation of the North Sea parasites. To test these assumptions, more populations from the hybrid zone gradient should be used in future experiments.

### **Sympatric vs. allopatric host–parasite combinations**

Texel parasites and hosts seem to have evolved mainly along the infectivity and resistance trait space, because parasites from Texel had the highest infection rates when infecting Sylt hosts, whereas Sylt parasites did not show significant differences among host sources. Therefore, Texel parasites must have evolved a higher infection potential in comparison to Sylt parasites that, in turn, might have been selected for higher specific resistance in their sympatric hosts. Evolution of host resistance was also observed in a reciprocal infection experiment with Japanese and European eels

and the invasive nematode *Anguillicola crassus*, where the sympatric combinations consistently had a lower infectivity than the allopatric combinations (Weclawski *et al.* 2013), indicating consistent resistance evolution of the host. However, *Mytilicola* infections had no consistent lower infectivity in sympatric combinations and this could be due to the invasion of the congener *M. orientalis*, which also infects mussels and is found on Texel but not in the northern part of Sylt (Elsner *et al.* 2010; pers. observation). Given that the host's resources are limited, co-infection by both parasites could lead to increased competition, which in turn may lead to selection for higher infectivity and more efficient exploitation by the parasite. However, *M. orientalis* only became common on Texel after the arrival of the invasive Pacific oyster on Texel (1983; Troost 2010), making an evolutionary response to higher competition less likely.

Sympatric infections were more virulent than allopatric ones, or sympatric hosts less tolerant, and we only found indirect evidence for differential evolution of post-infection traits, i.e. tolerance and virulence (Table 4.1B and C and Figure 4.4). In general, tolerance is more difficult to show experimentally (Raberg *et al.* 2009), since infectivity influences infection intensity, and thereby also our tolerance estimate. To properly investigate tolerance, a full range of infection intensities from all parasite sources is needed.

Indirectly, however, there are some indications for higher tolerance in Sylt hosts. Even though Sylt hosts had a higher infection rate by Texel parasites, and Texel parasites exploited Sylt hosts more than any other host–parasite combination, sympatric parasites caused an 18.75% reduction in dry weight in Sylt hosts. Texel hosts had equal infection rates for sympatric and allopatric parasites, and neither showed high exploitation efficiencies; nevertheless, the reduction of host dry weight in the sympatric infection combination was 28.21%, coupled with a steeper slope of the intensity vs. dry weight relationship ( $-0.013$  on Texel vs.  $-0.0032$  on Sylt). Therefore, indirectly, these results may indicate that Sylt hosts might be more tolerant to being parasitised by *M. intestinalis* than Texel hosts and that they evolved along the post-infection trait space. The specific patterns of pre- and post-infection traits in sympatric and allopatric combinations suggest that within the invasive population expansions towards Texel and Sylt, different evolutionary trajectories could be observed over approximately the same co-evolutionary time frame (~45 years,

corresponding to <100 generations). This means that co-evolution does not necessarily have a fixed outcome, but can vary depending on the ecological conditions throughout the invasion of a parasite. In other systems, however, such as the rabbit–*Myxoma* system or the eel–nematode system, the same evolutionary outcome was repeatedly observed in several invasions (Kerr *et al.* 2015), or patterns were at least more uniform than for the *M. intestinalis* system (Weclawski *et al.* 2013). This could indicate that the ecological conditions in these systems were also more uniform and thus selected repeatedly for the same trait combinations, whereas selective forces of *M. intestinalis* invasions might differ more substantially.

The observed differences in evolutionary trajectories along the two invasion waves of the parasite could be connected to the ecological conditions encountered throughout the invasion process. Hydrodynamic regimes differ between the mixed oyster–mussel beds, as the Sylt site is located within a protected bay, while the site on Texel is more exposed. The hydrodynamic shear could influence the number of *M. intestinalis* larvae (and parasites in general) that are retained within each host population. Prevalence data from both sites supports this idea (Goedknecht *et al.*, unpublished data). Since transmission is often traded off against virulence (Alizon *et al.* 2009; Frank 1996), selection on infectivity might be stronger for Texel parasites while the high number of infective stages on Sylt might select for tolerance in the host.

### **Disentangling host from parasite adaptation**

In the present study there was only little evidence for local adaptation of the parasite to their sympatric host. The reduction of host body condition observed in sympatric host–parasite combinations could be interpreted as local adaptation of the parasite that evolved to exploit its local host. However, if the observed pattern was indeed due to increased host exploitation, this should be reflected in faster parasite development or a larger parasite mean length in sympatric combinations in comparison to allopatric combinations. We neither observed faster development in sympatric host–parasite combinations (Figure 4.5B), nor a more efficient exploitation of the host’s resources in sympatric parasites (Figure 4.5C), although competition among parasites occurred at higher infection intensities (Figure 4.5A). Here, the negative correlation between parasite development rate and infection intensity (Figure 4.5A) could indicate that

resources within the hosts were limited, that there are host responses that are density-dependent, or that there is interference competition between parasites.

The reduction of host condition in sympatric combinations might reflect host adaptation and indicate that defence against parasite infection is costly. Increased specificity of immunological surveillance towards sympatric parasites might lead to better detection of the parasite and consequently divert more resources towards immunity or inflict more self-harm as a negative side effect of an increased immune response (Schmid-Hempel 2009). To conclusively demonstrate the mechanistic basis of these effects in the interaction between *M. intestinalis* and its sympatric host combinations, future experiments should measure the immune response and potential self-harming immune substances (e.g., reactive oxygen species) directly or indirectly by investigating the underlying molecular mechanisms (e.g., transcriptional responses of both partners; Greenwood *et al.* 2016).

## Conclusion

Non-native parasites are ideal candidates to reduce the existing biases in studies investigating host–parasite co-evolution. Regarding a bias towards microparasites, our study now highlights the suitability of invasive macroparasites for investigating co-evolution in nature. The possibilities to perform time-shift experiments on natural systems are restricted to few systems (Berenos *et al.* 2011a; Buckling & Rainey 2002; Decaestecker *et al.* 2007; Schulte *et al.* 2010), many of which rely on asexually reproducing hosts and parasites. However, as seen in our experiment, the effect of evolutionary time can be studied if invasive parasites are taken from different regions (space-for-time substitution) (Reusch 2014), especially if these represent different fronts of biological invasions. The use of host–parasite pairs from the native range could then further extend the range of co-evolutionary time from ancient to recent sympatry. In repeated invasion events or different invasion fronts one can further test whether host–parasite co-evolution leads to predictable outcomes (Wendling & Wegner 2015). Here, we can conclude that in the mussel–*Mytilicola* system independent invasions of parasites have led to different evolutionary trajectories on both the host and parasite side. Hosts on Texel seem to have evolved pre-infection traits, whereas hosts on Sylt may have evolved post-infection traits. This shows that

the evolutionary outcome of host–parasite interactions may not always be predictable, even within the same host–parasite system. The outcome of co-evolution therefore also depends on the ecological conditions in terms of the abiotic and biotic environment, which should be included into future studies of host–parasite co-evolution to gain a better understanding of co-evolution in nature.

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Dual transcriptomics reveals co-evolutionary  
mechanisms of intestinal parasite infections in blue  
mussels *Mytilus edulis*

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## Abstract

On theoretical grounds, antagonistic co-evolution between hosts and their parasites should be a widespread phenomenon but only received little empirical support so far. Consequently, the underlying molecular mechanisms and evolutionary steps remain elusive, especially in nonmodel systems. Here, we utilised the natural history of invasive parasites to document the molecular underpinnings of co-evolutionary trajectories. We applied a dual-species transcriptomics approach to experimental cross-infections of blue mussel *Mytilus edulis* hosts and their invasive parasitic copepods *Mytilicola intestinalis* from two invasion fronts in the Wadden Sea. We identified differentially regulated genes from an experimental infection contrast for hosts (infected vs. control) and a sympatry contrast (sympatric vs. allopatric combinations) for both hosts and parasites. The damage incurred by *Mytilicola* infection and the following immune response of the host were mainly reflected in cell division processes, wound healing, apoptosis and the production of reactive oxygen species (ROS). Furthermore, the functional coupling of host and parasite sympatry contrasts revealed the concerted regulation of chitin digestion by a Chitotriosidase 1 homolog in hosts with several cuticle proteins in the parasite. Together with the coupled regulation of ROS producers and antagonists, these genes represent candidates that mediate the different evolutionary trajectories within the parasite's invasion. The host–parasite combination-specific coupling of these effector mechanisms suggests that underlying recognition mechanisms create specificity and local adaptation. In this way, our study demonstrates the use of invasive species' natural history to elucidate molecular mechanisms of host–parasite co-evolution in the wild.

*Keywords:* biological invasion; coevolution; *de novo* transcriptomics; host–parasite interactions; immune response; macroparasite

## Introduction

Molecular evolution is a comparatively slow process, and rapid adaptation requires either a large amount of standing genetic variation (Barrett & Schluter 2008), or plastic mechanisms, for example, gene regulation or epigenetics (e.g., Rey *et al.* 2016; Shama *et al.* 2016). The interaction between hosts and parasites is a good example of rapid and dynamic evolution (Brockhurst *et al.* 2007; Buckling & Rainey 2002; Decaestecker *et al.* 2007). Here, the host can evolve resistance to prevent a parasite from infecting, or evolve tolerance to minimise the damage by the parasite (Schneider & Ayres 2008). The parasite, in turn, maximises its fitness by evolving infectivity and virulence leading to the antagonistic arms race between host and parasite, which is characterised by rapid reciprocal adaptation (e.g., Bishop *et al.* 2000). Although co-evolution is a key assumption of host–parasite evolutionary theory, empirical support is lacking far behind with only few studies supporting predictions of co-evolution. Most of these originate from the laboratory (e.g., Berenos *et al.* 2011b; Buckling & Rainey 2002; Eizaguirre *et al.* 2012) and very few come from the wild (Decaestecker *et al.* 2007). The phenotypes of characterizing these antagonistic interactions may appear similar, but the underlying molecular mechanism may differ substantially. Conversely, seemingly different phenotypic trajectories may also be based on the same molecular machinery that merely differs in its expression levels (Elmer *et al.* 2014; Hanson *et al.* 2017). Overall, our mechanistic understanding, and consequently also our understanding of the mode and speed of co-evolution, is so far limited.

Parasite invasions are ideal to study the temporal dynamics of host–parasite evolutionary interactions in the wild (Goedknecht *et al.* 2016). For instance, it is possible to use the implicit space for time substitution (Reusch 2014) of invasive parasites along their invasion route to study host–parasite co-evolution along a time axis covering ancient to no sympatry (*Chapter 4*, Feis *et al.* 2016; Weclawski *et al.* 2013). In this approach, hosts and parasites are taken from several locations along their invasion route for controlled cross-infection experiments. This setup can help elucidate how the duration of co-evolution influences the evolution of the host resistance and tolerance, as well as the parasite’s infectivity and virulence in the wild (Feis *et al.* 2016; Kelehear *et al.* 2012; Weclawski *et al.* 2013), without the need to raise multiple generations in the laboratory to use in time-shift experiments (i.e., in Berenos *et al.* 2011a; Brockhurst *et al.* 2007; Schulte *et al.* 2010). Unlike time-shift

experiments, however, hosts at invasion fronts are at the start of their co-evolutionary interaction, whereas parasites have been moving across the host population for some time near the invasion origin. It is nevertheless possible to test the mode of evolution along invasion pathways or to test for local adaptation, by taking hosts and parasites from separate locations along the historical invasion front, thus creating a sympatry vs. allopatry contrast. For instance, the same mode of parasite evolution after invasion took place repeatedly in rabbits infected with the Myxoma virus in Australia and the United Kingdom, leading to parallel changes in viral virulence across locations (Kerr *et al.* 2015). Experiments with invasive species can thus address questions about the adaptive evolution of phenotypes in nature, especially for nonmodel organisms with long generation times that prohibit experiments over multiple generations (Colautti & Barrett 2013).

The invasion of the parasitic copepod *Mytilicola intestinalis* into the Wadden Sea unites all aspects of an invasion to study host–parasite co-evolution (*Chapter 4*, Feis *et al.* 2016). Native in the Mediterranean and Adriatic Seas, *M. intestinalis* invaded mussel beds in the late 1930s (Caspers 1939) and has spread southwest towards the island of Texel (The Netherlands) (Drinkwaard 1993) and northward to Sylt (Germany) (Dethlefsen 1972), arriving at both islands around the same time in the early 1970s. The invasion routes are thus replicates of a newly begun co-evolutionary relationship between native blue mussel *Mytilus edulis* populations and the parasite *M. intestinalis*. Although the parasitic lifestyle of *M. intestinalis* was sometimes debated (Davey *et al.* 1977), we could already show that *M. intestinalis* and mussels have evolved different evolutionary trajectories along their invasion routes. Specifically, sympatric combinations of *M. intestinalis* and mussel hosts caused more damage to the host than allopatric combinations (*Chapter 4*, Feis *et al.*, 2016), probably owing to fact that *Mytilicola* parasites mainly consume host tissue (Goedknegt *et al.* 2018). Mussels from the southwest invasion front on Texel, for example, were more resistant than those from Sylt, while mussels from Sylt tended to be more tolerant to being parasitised (*Chapter 4*, Feis *et al.* 2016)

To understand the evolutionary process leading to the phenotypic differences, the molecular mechanisms in the mussel–*Mytilicola* relationship need to be deciphered. The major immune pathways are reasonably well characterised in bivalves and gastropods (Gerdol 2017). For mussels (*Mytilus* spp.), these were mainly derived from transcriptional and metabolite studies in response to microbial

pathogens, for example, Gram-positive and Gram-negative bacteria (Liu *et al.* 2014; Toubiana *et al.* 2014; Venier *et al.* 2011), yeast and filamentous fungi (Sonthi *et al.* 2012). Because -omics tools were essential in the description of important pathways in mussel immunity (Gerdol & Venier 2015), this approach can also be useful to characterise the immune response towards macroparasitic infections. Macroparasites have been targeted only by very few mussel studies (e.g., trematode infections, Gorbushin & Iakovleva 2011), despite a high parasite richness of this widespread epibenthic filter feeder (Thieltges *et al.* 2013). Methodologically, macroparasite infections have the advantage that host and parasite transcriptomes can be sequenced separately, extending a dual transcriptomics approach to a dual-species transcriptomics approach. In microbial infections, hosts and parasites often cannot be separated physically and classical dual transcriptomics relies on bioinformatic separation of host and parasite transcripts leading to heavy bias towards the host with only few parasite transcripts (Greenwood *et al.* 2016). Nevertheless, a dual transcriptomics approach can be very helpful to identify interacting genes (Schulze *et al.* 2016; Westermann *et al.* 2012) and has been applied to several model organisms (e.g., Choi *et al.* 2014; Pittman *et al.* 2014; Rosani *et al.* 2015; Tierney *et al.* 2012). For bivalves, the interaction between the Pacific oyster *Magallana gigas* (previously known as *Crassostrea gigas*, see Salvi & Mariottini 2017) and Ostreid Herpesvirus type 1 (OsHV-1) has been characterised using this approach, revealing that OsHV-1 interfered with active host defence by suppression of cytokine signalling through interferon-related pathways (Rosani *et al.* 2015).

So far, these dual transcriptomics studies have mainly focused on the infection process as such, but have not incorporated the geographic aspects of co-evolutionary rapid local adaptation or contrasted sympatric and allopatric combinations of hosts and parasites.

Here, we incorporate these aspects by combining the natural history of the *Mytilicola* invasion with a dual-species RNA-seq analysis of a reciprocal infection experiment. This approach can uncover the molecular underpinnings of the mussel–*Mytilicola* relationship at the transcriptional level and thus help explain the different evolutionary trajectories observed along the invasion route of *Mytilicola* (Chapter 4, Feis *et al.* 2016). We characterise this interaction on three different levels representing our explicit experimental contrasts. First, we ask which genes and processes are involved in regulatory responses of hosts infected by *Mytilicola* by

contrasting experimentally infected hosts with control hosts, representing the infection contrast. Second, we identify host genes that are differentially regulated in infections with locally co-evolved parasites compared to infections with parasites that lack a recent shared co-evolutionary history, representing the sympatry-allopatry contrast. Lastly, our dual-species transcriptomics approach allows us to use the same sympatry-allopatry contrast to identify differentially regulated genes in the parasite, which can be matched to the host sympatry-allopatry contrast, and couple phenotypic evolutionary trajectories to co-evolved gene regulatory processes in antagonistically interacting species.

## **Materials and methods**

### **Experimental procedures**

The details of the underlying cross-infection experiment are described in Feis *et al.* (2016) (*Chapter 4*). Briefly, mussels *M. edulis* and parasitic copepods *M. intestinalis* were collected at two locations, representing the two invasion pathways of the parasite. These were at Vlakte van Kerken on Texel, The Netherlands, representing the southwestern pathway and in Königshafen on Sylt, Germany, representing the northern pathway. The mussels used in the experiment were treated against any previous *Mytilicola* spp. infections according to the method of Bateau *et al.* (1992) and were left to recover after the anti-*Mytilicola* treatment for at least 2 weeks before the experiment started. The copepodites used in the experiment to infect mussels were hatched from egg sacks of gravid *M. intestinalis* from the two locations. Mussels were infected with 24 copepodites from one or two mothers. Therefore, copepodites within one infection were related; however, we do not know whether multiple males sired the offspring from one female. Assuming that relatedness within the population will be 0, we can be sure that relatedness was higher within a single infection than between infections. In a full-factorial design, we generated different experimental contrasts, that is, infected vs. uninfected control mussels, sympatric (Texel host × Texel parasite, Sylt host × Sylt parasite) and allopatric (Texel host × Sylt parasite and vice versa) host and parasite combinations. In the control treatment, we did not expose mussels to any copepodites. Each mussel was kept in a temperature-controlled

room at 18 °C in its own bottle that was supplied with its own flow-through inlet distributing filtered seawater equally (salinity of the water input was 29–30 psu). Bottles with mussels were randomly relocated within and between containers twice per week. Mussels were dissected under a stereomicroscope 80 days after the experiment had started. The intestinal tissue of each mussel, which is in direct contact with the parasite, and *M. intestinalis* specimens were put in RNAlater (Qiagen, Hilden, Germany) separately and were frozen at 80 °C until further processing.

### RNA extraction, library preparation and sequencing

RNA extractions of mussels and parasites were done with the RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). Since extraction of single female parasites did not yield enough RNA for sequencing, we pooled three female *M. intestinalis* per sample. We had three biological replicates for each treatment combination (see Table 5.1), except for the Texel host × Texel parasite combination, for which we had two biological replicates as not enough material was present for pooling a third replicate. Total RNA concentrations were measured on a NanoDrop ND-1000 spectrophotometer (Peqlab [VWR], Erlangen, Germany). The concentration, purity and integrity of the RNA were further measured and checked with an Agilent 2100 Bioanalyzer using the Agilent RNA 6000 Nano Kit (Agilent Technologies, Waldbronn, Germany). Library preparation was done with the TruSeq Stranded mRNA HT Library Prep Kit (Illumina, San Diego, CA, USA). We used an Agilent 2100 Bioanalyzer with the Agilent 7500 Nano Kit (Agilent Technologies, Waldbronn, Germany) to quantify the amount of cDNA in each library and to check the quality of each cDNA library, after which the cDNA libraries were pooled equimolarly. All of the above kits were used according to the manufacturer’s protocols. Next-generation sequencing was performed on a Next-Seq500 DNA sequencer (Illumina, San Diego,

**Table 5.1** Overview of the number of replicates successfully sequenced for each experimental treatment. For the parasite RNA-seq data, the first number represents the number of biological replicates and the second number the amount of parasites pooled within each replicate.

	Host RNA-seq data			Parasite RNA-seq data	
	Texel <i>Mytilicola</i> <i>intestinalis</i>	Sylt <i>M. intestinalis</i>	No parasite	Texel <i>M. intestinalis</i>	Sylt <i>M. intestinalis</i>
	Texel mussel	3	3	3	2×3
Sylt mussel	3	3	3	3×3	3×3

CA, USA) at the Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research with a single-end sequencing strategy at a read length of 75 bp. Illumina BCL files were converted to fastq files and demultiplexed using bcl2fastq (v2.17, Illumina, San Diego, CA, USA) with default settings. Trimmomatic (Bolger *et al.* 2014) was used to clean raw reads by filtering out short (<36 bp) and low-quality reads (sliding window option), as well as adapters if still present.

### ***Mytilus edulis*: assembly and annotation**

Reads from 18 *M. edulis* gut libraries (BioSample Accession nos. SAMN08395021-SAMN08395038 in BioProject PRJNA430138) presented here were combined with reads from a further 27 *M. edulis* gut libraries (BioSample Accession nos. SAMN08395039-SAMN08395065 in Bioproject PRJNA430138) for *de novo* assembly of a mussel transcriptome backbone. All libraries were derived from the same experiment with the same protocols. We chose to make transcriptome backbones with all libraries for assembly robustness and analysed only the first subset of 18 *M. edulis* libraries that were relevant for the current question. The backbone was assembled *de novo* in CLC Genomics Workbench 8.5.1 (CLCbio, Denmark) with default parameters, using automatic word and bubble size and a minimum contig size of 200 nucleotides. Contigs were updated by back-mapping of reads (mismatch cost 2, insertion and deletion cost 3, length fraction 0.5 and similarity fraction 0.8). The mussel transcriptome backbone was annotated using the Trinotate pipeline (Grabherr *et al.* 2011), including BLAST (Altschul *et al.* 1990) and Gene Ontology (GO) annotation (The Gene Ontology Consortium, 2000).

### ***Mytilicola intestinalis*: assembly and annotation**

Reads from 11 *M. intestinalis* libraries (BioSample Accession nos. SAMN08394988 – SAMN08394998 in BioProject PRJNA430138) presented here were combined with reads from 13 *M. intestinalis* libraries (BioSample Accession nos. SAMN08394999 – SAMN08395006, SAMN08395008 and SAMN08395010 in BioProject PRJNA430138) from another experiment for *de novo* assembly of a parasite transcriptome backbone in CLC Genomics Workbench 8.5.1 (CLCbio, Denmark) with identical parameters used for the mussel assembly (see above). All libraries were derived from the same experiment with the same protocols, but only the 11 *M. intestinalis* libraries presented here were relevant for the question in this manuscript.



The parasite transcriptome backbone was annotated by BLAST of the contig sequences against UniRef, only retaining hits with an e-value cut-off of  $<1 \times 10^{-5}$ . We used TransDecoder to find the most probable ORFs (Haas *et al.* 2013) and InterProScan (Jones *et al.* 2014) to gain more insight into the potential function of differentially expressed *M. intestinalis* contigs without a BLAST annotation. After removing contigs that had a bivalve (host) or phytoplankton (food) annotation, 44,616 contigs remained (see Table 5.2 for assembly statistics). The quality of the parasite transcriptome backbone was assessed through BUSCO (Simao *et al.* 2015) (Table S5.1).

The same gene in different organisms may have different functions, but belong to the same gene regulatory network (e.g., Hinman & Davidson 2007). Understanding the potential pitfalls of inferring function from annotations based on sequence similarity without further experimental evidence on each specific gene for mussel and *M. intestinalis*, we will assume homologous function for the differentially expressed genes we report.

### **Differential expression analyses**

Differential expression analyses and GO enrichment analysis were performed in the R statistical environment version 3.4.1 (R Core Team 2017), after the reads from each library were mapped back to the respective transcriptome backbone, using the RNA-seq tool in CLC Genomics Workbench 8.5.1 (CLCbio, Denmark), with the length and similarity fraction parameters set to 0.5 and 0.8, respectively. The resulting count data were further analysed using the DESeq2 package (Love *et al.* 2014) from BioConductor (Huber *et al.* 2015), where we selected the 18 transcriptomes of mussels and 11 of parasites that belong to the experimental groups described in this article.

To address the different questions for mussel hosts, we first analysed the complete mussel RNA-seq data set to examine the difference between all infected vs. all control mussels and then focused on a subset including only the infected mussels to differentiate between sympatric and allopatric infections. For the parasite, we only contrasted sympatric and allopatric infections. The DESeq experiment design was full rank for all data sets. To include only meaningfully expressed contigs, we applied a mapping cut-off filter by excluding contigs with fewer than five hits per sample on average. Main effects and interaction effects were tested by Wald tests. For each main

and interaction effect, we used the *fdrtool* for false discovery rate correction with the Benjamin-Hochberg method (Strimmer 2008a, b). We consider contigs differentially expressed if the adjusted *p*-value ( $p_{adj}$ ) < 0.05 and the absolute log2fold change (LFC)  $\geq 1.5$ .

To gain further insights into the functions of differentially expressed contigs for mussels, we performed GO enrichment analyses for biological processes of the main and interaction terms based on the GO annotations from Trinotate using the R-package *topGO* (Alexa *et al.* 2006). A similar approach for *M. intestinalis* was not successful because of the low number of differentially expressed contigs with GO annotations. Due to their functional relevance in the interaction with *M. intestinalis*, we examined the differentially expressed mussel genes with the GO annotations (including offspring) in the biological processes of “response to stimulus” (GO:0050896), “immune system processes” (GO:0002376) and “death” (GO:0016265) closer, and in addition, we manually curated this DEG set by including contigs with the same or similar protein name that lacked a GO annotation.

## Results

### Mussel and *Mytilicola* transcriptome backbone quality

We obtained a total of 348 million cleaned reads for the mussel *M. edulis* and 108 million cleaned reads for the parasite *M. intestinalis*, resulting in assemblies of 163,270 and 45,645 contigs, respectively (Table 5.2). Applying our mapping cut-off

**Table 5.2** Summary of sequencing, assembly and annotation statistics.

	<i>Mytilus edulis</i>	<i>Mytilicola intestinalis</i>
<b>Sequencing statistics</b>		
Number of clean reads	347,996,951	108,116,930
Total bases	26,215,385,329	8,144,066,749
% GC content	40.8%	50.2%
<b>Assembly statistics</b>		
Number of contigs	163,270	45,645
Average contig length	641	738
N50 contig length	1,206	1,289
Range of contig lengths	200 – 13,745	200 – 19,927
<b>Annotation statistics</b>		
Annotated contigs by blastx	29,799 (18.3%)	12,653 (27.8%)
Annotated contigs by blastp	22,451 (13.8%)	16,259 (35.7%)
Annotated contigs with GO	28,764 (17.6%)	12,826 (28.1%)
TOTAL annotated contigs	30,514 (18.7%)	16,259 (35.7%)

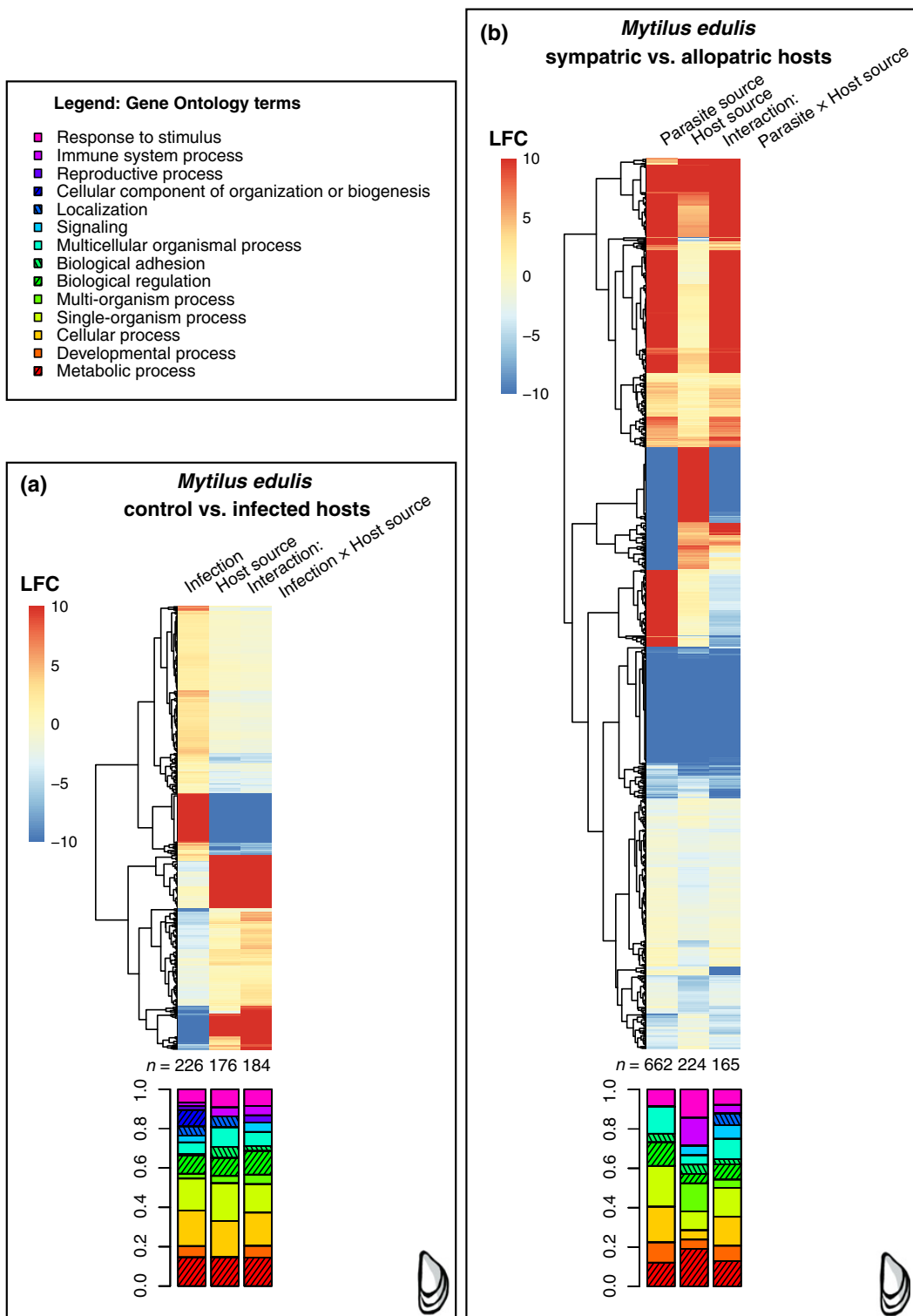
N50 = 50% of the bases are in contigs larger than this number; GO = Gene Ontology.

filter resulted in 63,577 contigs for the infection vs. control differential expression analyses and in 64,759 mussel and 19,546 parasite contigs for the sympatry vs. allopatry contrast.

### **Differential expression analysis in mussels: infection vs. control**

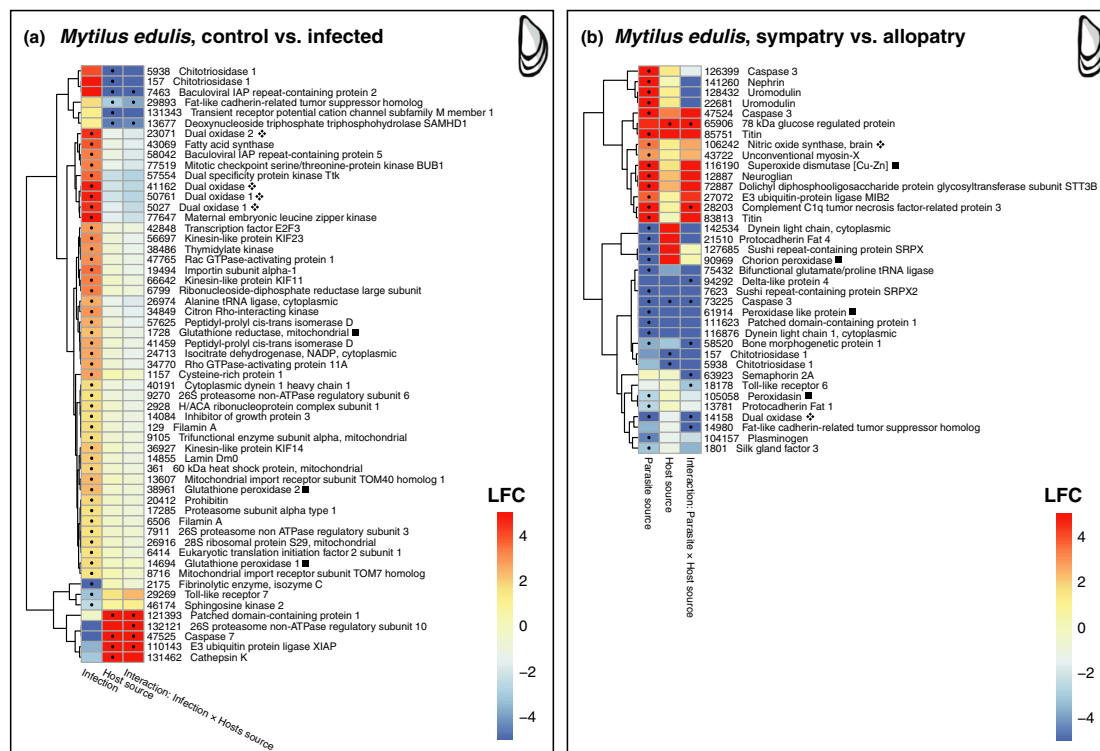
We found a total of 471 differentially expressed contigs ( $p_{\text{adj}} < 0.05$  and  $\text{LFC} \geq 1.5$ , 233 had an annotation, Table S5.2): 226 in the infection contrast, 176 in the host source contrast and 184 for the interaction infection by host source (Figure 5.1a, Table S5.2). Of those genes, 116, 30 and 17 in the respective contrasts belonged to significantly enriched biological processes identified by GO enrichment analyses. The most represented GO terms enriched by infection with *Mytilicola* belonged to cellular process (111 genes, GO:0009987, Fisher's exact test,  $p = 0.0286$ ), metabolic process (90 genes, GO:0008152, Fisher's exact test,  $p = 0.0125$ ), single-organism process (100 genes, GO:0044669, Fisher's exact test, n.s.) and biological regulation (57 genes, GO:0065007, Fisher's exact test, n.s.). Significantly enriched offspring terms of cellular process and single-organism process were mitotic cell cycle (GO:0000278, Fisher's exact test,  $p = 9.3\text{e-}06$ ) and cell division (GO:0051301, Fisher's exact test,  $p = 0.00023$ ). The GO process cellular component of organization or biogenesis (GO:0071840) was only found in the infection contrast. Its significant offspring terms included mitotic nuclear division (GO:0007067, Fisher's exact test,  $p = 6.33\text{e-}06$ ), ribosome biogenesis (GO:0042254, Fisher's exact test,  $p = 0.0116$ ) and mitotic spindle assembly (GO:0090307, Fisher's exact test,  $p = 0.000987$ ). Another significantly enriched term in the infection treatment was wound healing (GO:0042060, offspring of response to stimulus, Fisher's exact test,  $p = 0.0166$ ), suggesting that tissue damage caused by the parasite induced a tissue regeneration response. Offspring of the term reproductive process (GO:0022414) was significantly enriched in the infection contrast (12 genes) and in the interaction contrast (three genes), suggesting a resource shift from reproduction to wound healing when infected. Furthermore, the GO terms reactive oxygen species (ROS) metabolic process (GO:0072593) and response to oxidative stress (GO:0006979) were significantly enriched in the infection contrast (Fisher's exact tests,  $p = 3.2\text{e-}05$  and 0.00596, respectively).

Additionally, exploration of the data set including only the genes involved in immune system processes (GO:0002376), response to stimulus (GO:0050896) and



**Figure 5.1** Heat map of all differentially expressed *Mytilus edulis* contigs with a  $LFC \geq 1.5$  and  $p_{adj} < 0.05$  of **(a)** control vs. infected hosts (left column), between both host populations (centre column) and the interaction term (right column). **(b)** Sympatry-allopatry contrast with differential regulation between parasite (left column), hosts (centre column) and the interaction term (right column). For clarity, LFC values were capped at 10 and  $-10$ . Numbers of differentially regulated genes, as well as proportional classifications of Gene Ontology biological process terms, are given for each experimental contrast. A list of differentially expressed contigs of *M. edulis* (471 in the infection vs. control contrast; 946 in the sympatry vs. allopatry contrast) including annotations can be found in the online supplementary materials.

death processes (GO:0016265) resulted in a list of 46 differentially regulated genes (Figure 5.2a). Most notably, infection led to an upregulation of genes inducing ROS, such as four dual oxidase homologs (LFC ranging 4.4 – 4.8), as well as ROS antagonists, such as homologs of glutathione peroxidase 1 and 2 (LFC = 1.7 and 2.4, respectively) and glutathione reductase (LFC = 2.3). This is a further indication that ROS pathways are among the most prominent immune mechanisms against *M. intestinalis* infections. As a possible consequence of increased ROS damage, we also found upregulation of genes involved in apoptosis, including stimulators such as homologs of inhibitor of growth protein 3 (LFC = 1.6) and peptidyl-prolyl cis-trans isomerase D (LFC = 2.5 and 2.6; these two contigs are probably fragments of the same transcript, because the protein sequences did not overlap upon alignment, data not shown), as well as homologs of the inhibitors filamin A (LFC = 1.7 and 1.6; these two contigs showed overlap in their alignment, data not shown), KIF14 (LFC = 2.1), and baculoviral IAP repeat-containing protein (LFC = 3.3). Some of the immunity-



**Figure 5.2** Selection of contigs annotated with the Gene Ontology (GO) terms for biological processes including offspring GO terms: response to stimulus (GO:0050896), immune system processes (GO:0002376) and death (GO:0016265) that are involved in defence/immunity reaction to *Mytilicola intestinalis*. Dots in the heat map denote for which contrast(s) the contigs were significant ( $p_{adj} < .05$  and LFC  $\geq 1.5$ ). **(a)** Control vs. infected hosts. **(b)** Hosts infected with sympatric vs. allopatric parasites. Symbols denote ROS-producing genes (♦) and ROS antagonists (■). LFC values were capped at 5 and -5 for clarity.

related genes were downregulated in infected mussels (Figure 5.2a). The downregulation of a homolog of Toll-like receptor 7 (TLR7, LFC = -3.2), a key component of innate immunity which recognises molecular patterns specific to microorganisms, might indicate a shift of the immune response away from the Toll pathway (Toubiana *et al.* 2014). A homolog of sphingosine kinase 2 (LFC = -2.4), which may have pro- and anti-inflammatory functions (Neubauer & Pitson 2013), was also downregulated in *M. intestinalis*-infested mussels.

### **Differential expression analysis in mussels: sympatry vs. allopatry**

In comparison with the infection contrast, we found more than twice as many differentially expressed contigs (946 at  $p_{\text{adj}} < 0.05$  and  $\text{LFC} \geq 1.5$ , of which 262 were annotated, Table S5.3). The majority (662 genes, 70%) of these were caused by the different parasite sources, while only 224 (24%) were differentially expressed between the host sources. Host by parasite combinations led to specific induction patterns in relatively few genes (165 genes, 17%, Figure 5.1b), indicating that the combination-specific phenotypic patterns (Feis *et al.* 2016) rely on the regulation of relatively few genes. Within these respective contrasts, 27, 5 and 21 genes belonged to significantly enriched biological processes in the GO enrichment analyses of the contrasts among different parasite sources, different host sources and host by parasite combinations, respectively. The term immune system process (GO:0002376) was not significantly enriched (nor any of its offspring terms, Figure 5.1b) in the parasite contrast GO enrichment analyses, but it was in both host contrast and host 9 parasite contrast (Fisher's exact test,  $p = 0.00985$  and  $0.0436$ , respectively). Other significantly enriched GO terms in the host 9 parasite contrast were defence response (GO:0006952,  $p = 0.0225$ ), response to other organism (GO:0051707,  $p = 0.0128$ ) and response to bacterium (GO:0009617,  $p = 0.0037$ ), all of which are offspring terms of response to stimulus (GO:0050896).

The important role of parasite origin for host transcription becomes even more obvious in the curated data set that only contained genes involved in immune system (GO:0002376), response to stimulus (GO:0050896) and death processes (GO:0016265). Infection with the parasites from different sources changed regulation patterns in 30 out of 37 genes (81%), many of which were either stimulators or antagonists of ROS production (13 differentially expressed contigs: contig numbers 23,071, 41,162, 50,761, 5,027, 106,242 and 14,158 for ROS and contig numbers

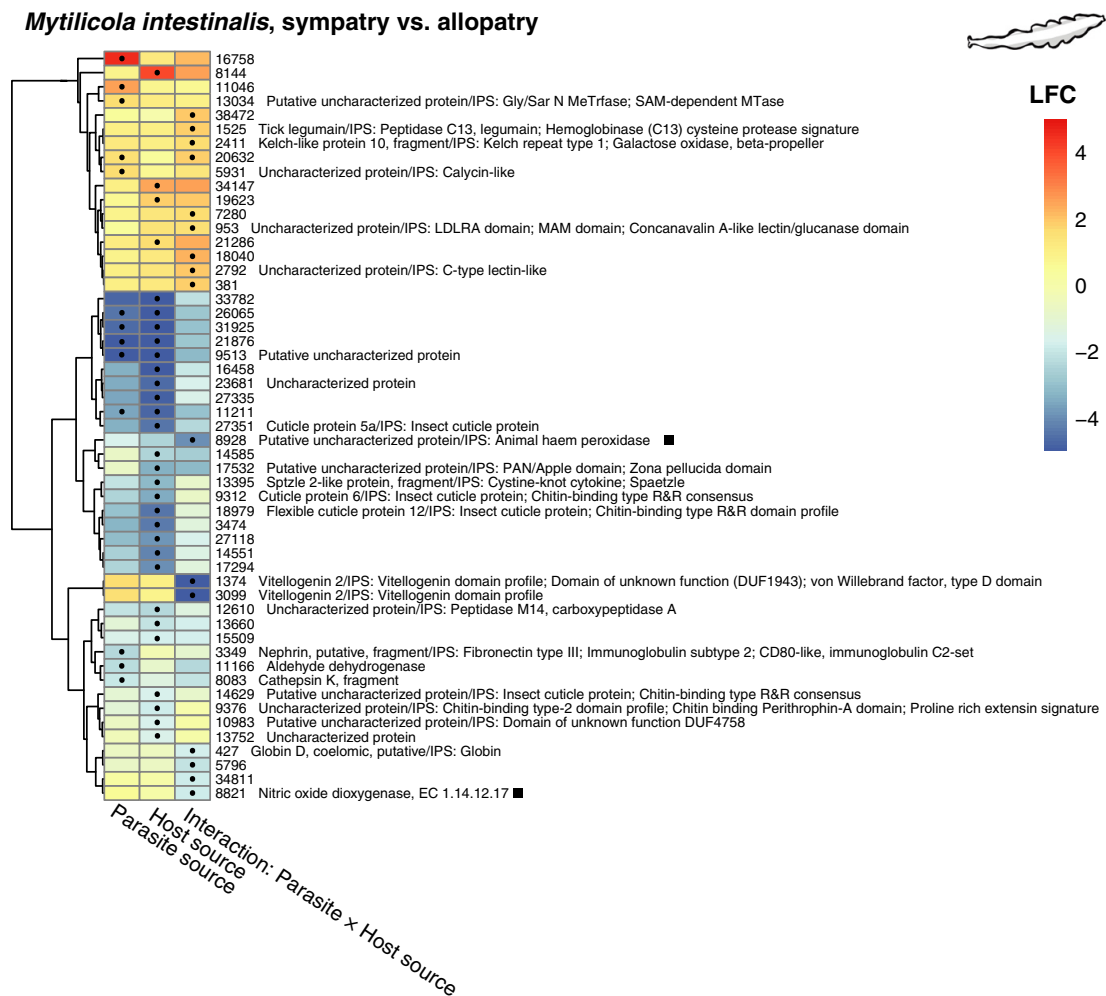
1,728, 38,961, 14,694, 116,190, 90,969, 61,914, 105,058 for ROS antagonists). Regulation of these genes was not uniform, but varied depending on the parasite origin. A superoxide dismutase [Cu-Zn] homolog (SOD, a ROS antagonist) was upregulated (LFC = 15.5) only in the mussels infected with Sylt parasites, while homologs of chorion peroxidase, peroxidase-like protein, peroxidasin (all antagonistic to ROS) and a dual oxidase homolog stimulating ROS production were all down-regulated (LFC = -20.5, -21.2, -2.7, -6.7, respectively). Similarly, several caspase 3 apoptosis homologs showed parasite-specific induction (contig numbers 126,399, LFC = 24.2; 47,524, LFC = 20.2; and 73,225, LFC = -19.6), suggesting that the regulation of genes with similar function can fine-tune functional responses at the phenotypic level. For the host contrast, only four genes (11%) were differentially regulated in the curated data set, most notably containing two Chitotriosidase 1 (Chit1) homologs that were less expressed in Sylt hosts (Figure 5.4). Chit1 is an enzyme that breaks down chitin. Both Chit1 contigs probably belong to the same gene, because no overlap existed between the aligned sequences (data not shown). Among the contigs significant in the host  $\times$  parasite contrast (i.e., sympatry contrast, the interaction term in the model), we found nine genes with combination-specific regulation patterns (Figure S5.2). These included the apoptosis factor homologs of C1q TNF3, Toll-like receptor 6 (TLR6), Caspase 3 and Fat-like cadherin-related tumour suppressor (Figure 5.2b, LFC = 6.7, -3.17, -25.8 and -23.5, respectively). Apart from several dual oxidase homologs (contig numbers 23,071, 41,162, 50,761, 5,027 and 14,158) that stimulate ROS production and reacted to infection in general (Figure 5.1a; these contigs all showed overlap between the aligned sequences, data not shown), we also found a dual oxidase homolog that was specifically upregulated in sympatric infections compared to allopatric infections (contig number 14,158, LFC = -13.0).

### **Differential expression analysis in *Mytilicola* parasites**

We found 53 differentially expressed contigs ( $p_{\text{adj}} < 0.05$  and  $\text{LFC} \geq 1.5$ ). In contrast to the host sympatry contrast, where most genes were differentially regulated depending on parasite source, in the parasite transcriptomes most differentially regulated genes were found between host sources (30 genes, 57%), while only 13 (25%) originated from differences between the parasites themselves. The interaction term representing regulation patterns for specific host 9 parasite combinations yielded

16 genes (30%). About half (26 genes) of the 53 differentially expressed genes had a significant BLAST or InterProScan hit, leaving 27 genes without putative functional annotation (Figure 5.3, Table S5.4).

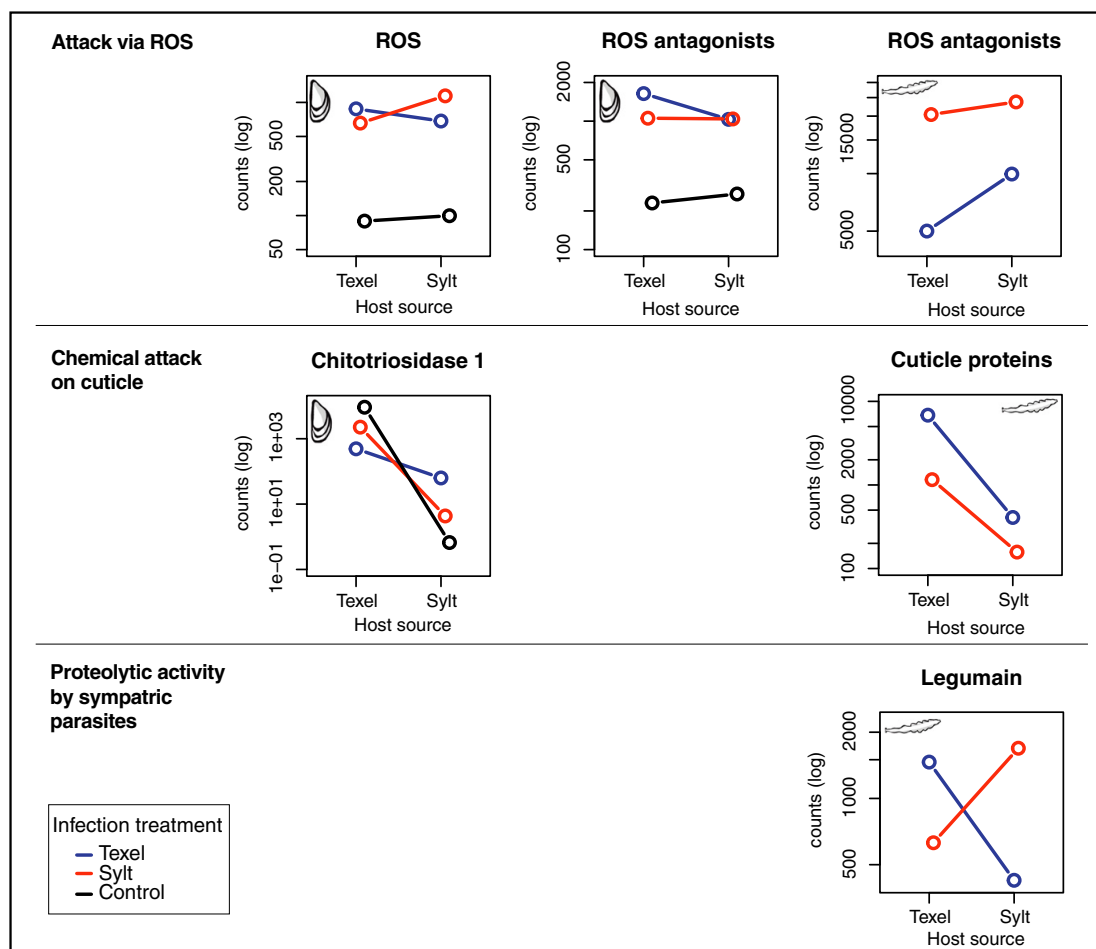
Differences between the host sources were mainly due to down-regulation of genes in the parasites infecting Sylt hosts (Figure 5.3). Where homologies could be determined, these mainly included genes involved in development of the cuticle (four cuticle proteins, one chitin-binding domain, LFC range  $-1.8$  to  $-4.4$ ; Figure S5.1), which could indicate that the external physical barrier of the parasite suffered less damage in the guts of Sylt mussels – a pattern matching the downregulation of Chitotriosidase 1 in Sylt mussels (Figures 5.2, 5.4 and S5.2).



**Figure 5.3.** Heat map of all differentially expressed *Mytilicola intestinalis* contigs. Dots in the heat map denote in which contrast(s) each of the contigs were significant ( $p_{adj} < 0.05$  and  $LFC \geq 1.5$ ). The annotation for each contig number as BLAST hit and InterProScan (IPS) homology are given wherever available. Symbols denote ROS producing genes (◇) and ROS antagonists (■). LFC values were capped at 5 and  $-5$  for clarity.



Out of the 13 differentially regulated genes between parasite sources, only three contigs downregulated in Sylt parasites had annotations: nephrin (contig number 3,349, LFC = -2.3), which plays a role in the development and function of kidneys in mice, aldehyde dehydrogenase (contig number 11,166, LFC = -2.3), which oxidises aldehydes and may be involved in lipid peroxidation, and cathepsin K (contig number 8,083, LFC = -2.0), a protease with a high specificity for kinins (Figure 5.3). These genes are involved in catabolic processes and suggest differences in breakdown processes of complex molecules between the parasite sources.



**Figure 5.4.** Functionally coupled genes between hosts and parasites. The upper panel shows the sums of ROS producing genes and ROS antagonists of hosts (six contigs numbered 23071, 41162, 50761, 5027, 106242 and 14158 for ROS; seven contigs numbered 1728, 38961, 14694, 116190, 90969, 61914, 105058 for ROS antagonists) and parasites (two contigs numbered 8928 and 8821). The middle shows attack on cuticle with the read counts of Chitotriosidase 1 of the hosts (both contigs) and cuticle proteins of the parasite (five contigs numbered 27351, 14629, 9376, 18979 and 9312). The lower panel shows proteolytic activity of the parasite (read counts of tick legumain) as a potential virulence factor. Interaction plots of the individual differentially regulated genes can be found in Figure S5.1, S5.2. Note different scales on y-axes used to illustrate the relative differences within rather than the absolute differences between species.

Combination-specific regulation patterns in parasite genes led to upregulation of nine genes in sympatric combinations. These included homologs of Tick legumain (LFC = 1.8; Figure S5.1) putatively involved in the digestion of host macromolecules in other parasites, Kelch-like protein 10 (LFC = 1.7), involved in fertility, as well as a C-type lectin-like (LFC = 2.0), involved in sugar binding/immune recognition. As a direct link to host gene regulation, ROS antagonists (nitric oxide dioxygenase, LFC = -1.8; animal haem peroxidase, LFC = -4.0) of parasites were specifically downregulated in sympatric combinations (Figures 5.3 and S5.1), which matches the parasite-specific regulation of nitric oxide synthase in the host and might contribute to the lower condition of host in sympatric infections (Figure 5.2) (*Chapter 4, Feis et al. 2016*). Parasites in sympatric combinations also seemed to allocate fewer resources to reproduction as indicated by the significant downregulation of two vitellogenin 2 homologs (Figures 5.3 and S5.1, LFC range -9.0 to -11.4).

## Discussion

Our dual-species transcriptomic analyses clearly showed that the transcriptional response of both hosts and parasites was mainly driven by the differences between the respective antagonists. The parasite's transcription mainly reacted to host origin, while host transcription reacted to the parasite origin. The resulting transcriptional response may thus be interpreted as a clear sign of co-evolutionary adjustment of gene expression in this host-parasite interaction. While our analysis only captures a single time point during the interaction of mussels and *Mytilicola*, we are confident that the chronic state of infection represents a relevant time point that integrates the interaction between host and parasite over an extended period. Adult female *M. intestinalis* cause the highest energy drain and can be found throughout the year in the field (F. Demann, unpublished data). In this way, we probably captured the chronic effects of the parasite rather than the acute ones, but such chronic effects can also be expected for the natural situation. At the functional level, we found that with 13 out of 37 differentially expressed annotated immune genes ROS pathways were important in the interaction between *M. intestinalis* and its mussel host. Because the ROS response is a fairly general and unspecific response to stress and diseases (Naviaux 2012), there have to be gene regulatory mechanisms that create the observed specificity reflecting

local adaptation by coupling of the matching host and parasite genes. Here, the higher expression of ROS-producing genes in sympatric hosts was coupled to ROS antagonist production in the corresponding parasites demonstrating this specificity (Figures 5.4, S5.1, and S5.2). This link between host and parasite transcriptomes was also reflected in other functionally related interactions. For example, chitin digestion of the hosts was coupled to cuticle protein expression in the parasite and might be a core mechanism underlying the resistance phenotype differences previously observed along the invasion fronts (*Chapter 4*, Feis *et al.* 2016). Such coupled gene expression pattern not only demonstrates the usefulness of dual transcriptomics approaches (Greenwood *et al.* 2016), but, more importantly, it shows that regulation of functionally important differences evolved rapidly along the two invasion fronts, supporting the usefulness of invasive species' natural history to understand host–parasite co-evolution in the wild.

#### **Mussel's response to infestation by *M. intestinalis***

Mussels that were experimentally infected with *M. intestinalis* parasites regulated the expression of genes with functions in cell cycling and cell division and associated with wound healing (Figure 5.1a). *Mytilicola intestinalis* is known to obstruct the mussel's intestine and perforate the intestinal wall with its appendages and hooks (Figueroa *et al.* 1991; Moore *et al.* 1977; Robledo *et al.* 1994), and this wounding is reflected in the transcriptome of the host. Increased cell division suggests that wounds in the gut wall are being closed and the intestinal epithelium is being replaced after disruption to guarantee proper function of the gut. In addition, downregulation of anticlotting agents (e.g., a homolog of fibrinolytic enzyme isozyme C) in *M. intestinalis*-infested mussels indicates an increased rate of blood clotting in infected mussels. Wounding and repair of physical damage therefore represent the major regulators of host gene expression.

Apart from the primary response to physical damage, host transcriptomes in the sympatry-allopatry contrast were also enriched in genes linked to an immune response. Similar to other studies investigating immune responses in mussels (e.g., Liu *et al.* 2014), we found the upregulation of genes involved in the production of ROS in *M. intestinalis*-infested mussels. This was accompanied by upregulation of genes providing antioxidants, likely to limit the negative effects of ROS on the host's own tissues. This mechanism of defence is indeed general and does not only combat

pathogen infections, but also plays a role in defence against infection by macroparasites (for instance, see Coustau *et al.* 2015). However, we additionally found significant differences in ROS response between sympatric and allopatric combinations. Therefore, although the ROS response may be a general reaction to infections, there is likely a certain degree of specificity in the regulation of these processes leading to local adaptation.

We further found a differential regulation of the homolog maternal embryonic leucine zipper kinase, an enzyme that can be involved in apoptotic processes (Jiang & Zhang 2013). Apoptosis may in part be a consequence of ROS production (Matés & Sánchez-Jiménez 2000). Apoptotic processes play an important role in the immune reaction to foreign substances and were predicted to be important as first line of defence in the digestive tissues of mussels to macroparasites such as *M. intestinalis* (Romero *et al.* 2015). Consequently, apart from the direct response to wounding, the upregulation of genes associated with cell division processes may also be linked to apoptosis by replacing apoptotic cells.

Other immune genes that were differentially expressed in infected mussels (Figure 5.2a) were either annotated by host–virus interaction according to the UniProt database (BUB1, SAMDH, importin subunit alpha-1) (Foulger *et al.* 2015; Masson *et al.* 2014; The UniProt Consortium 2017), or were related to response to microorganisms. Intestinal parasites have indeed been shown to alter microbial gut homeostasis (e.g., Berrilli *et al.* 2012; Cariveau *et al.* 2014; Kreisinger *et al.* 2015). The downregulation of TLR7 in infected hosts may suggest a shift away from bacterial defence pathways (such as the Toll pathway) in *M. intestinalis*-infested mussels. Combined with the constant wounding of the gut epithelium, this might lead to a higher susceptibility towards secondary infections.

Chronic infection by *M. intestinalis* therefore orchestrates a multilayered cascade of transcriptional reactions in the mussel host, involving ROS mediated and apoptotic immune responses, both potentially damaging to host tissues. Together with the physical damage caused by lesions in the gut wall, these may lead to substantial wounding and cell division response to maintain gut homeostasis.

### **Dual transcriptomics: coupled gene expression with links to phenotype**

The putative function of differentially regulated genes in the parasite was associated with metabolic processes such as proteolytic digestion. Here, especially the

combination-specific expression pattern of the tick legumain homolog (contig number 1,525) could be a marker for parasite feeding activity on host tissue. Legumain is expressed in the midgut in blood digesting ticks, where it serves as a haemoglobinase, processing haemoglobin and activating other proteases (Dall & Brandstetter 2016; Sojka *et al.* 2011). In other invertebrate groups (Fuzita *et al.* 2015 and references therein), but also in the protochordate *Branchiostoma belcheri* (Teng *et al.* 2009), legumain plays a role in food digestion: it breaks down macromolecules in the acidic gut environment. Thus, the higher legumain expression levels in sympatric combinations could be a direct indicator of lower host condition (Feis *et al.* 2016). Furthermore, other genes potentially involved in feeding (e.g., MAM and a low-density lipoprotein [LDL]-receptor class A [LDLRA] domain-containing protein) showed similar host–parasite combination-specific expression patterns, indicating once more a stronger exploitation of sympatric hosts. Arthropods have to acquire cholesterol from their diet as they are incapable of its de novo biosynthesis (Hassett 2004; Perner *et al.* 2016), LDL is a rich source of cholesterol (Perner *et al.* 2016). Consequently, LDL receptors are also upregulated in scorpion digestion (Fuzita *et al.* 2015). The increased transcription of these genes in sympatric combinations might therefore indicate increased digestion of host tissue by *Mytilicola*, leading to higher energy drain from the host, which could further contribute to lower body condition in sympatric combinations (*Chapter 4*, Feis *et al.* 2016).

Other genes with host–parasite combination-specific expression patterns in the sympatry contrast were involved in the parasite’s immune response or defence against the host’s immune response. The upregulation of a C-type lectin homolog in sympatric combination (Figure S5.1) suggests an increased immune activity of the parasite itself. C-type lectins are widespread pattern recognition molecules that are involved in carbohydrate recognition often associated with bacterial lipopolysaccharide in crustaceans (Kawabata & Iwanaga 1999), suggesting that the parasite’s immunological surveillance may be fine-tuned towards bacteria encountered in sympatric host guts. Since immune resources of the host are diverted to combat the parasite, less immune resources might be available to maintain bacterial homeostasis in the gut. Disturbed host microbiota can contain higher number of secondary opportunists (Lokmer & Wegner 2015) that could potentially also harm the parasite. This effect might be particularly strong in the already weakened sympatric

hosts therefore calling for an increased level of immunological surveillance on the parasite's side as well.

A strong relative increase in host ROS-producer transcription after infection (Figure 5.4) was one of the main defence mechanisms. Here, the pattern of host ROS-producer transcription supports the hypothesis of a higher degree of self-damage in sympatric combinations (*Chapter 4, Feis et al. 2016*). Host expression patterns of ROS antagonists, on the other hand, displayed opposite patterns, with especially high levels of host ROS antagonist expression in Texel hosts  $\times$  Texel parasites combinations (Figure 5.4). However, the parasite expression profiles revealed that the parasites in Texel host  $\times$  Texel parasite combinations hardly express any ROS antagonists. Assuming that transcription will positively correlate with ROS production this may indicate that the total amount of ROS scavengers might be lower and more ROS is freely available. A qualitatively similar pattern could be derived for the combination of Sylt hosts  $\times$  Sylt parasites, where the increase in host ROS production expression was not associated with a remarkable increase in either host or parasite ROS antagonist expression, which might also result in a higher relative amount of freely available ROS in the mussel gut. The high level of ROS antagonist transcription in Sylt parasites in general might therefore explain the small reduction in host condition following infection with parasites from this location (*Chapter 4, Feis et al. 2016*). The link between ROS production and ROS antagonist expression couples the expression patterns of parasites to those of the host and may be associated with to the phenotype of lowered body condition potentially resulting from oxidative stress. Further studies should therefore measure ROS production and oxidative stress directly to test this link on a functional level (Griendling *et al.* 2016).

Apart from the expression patterns of ROS pathways in the interaction between host and parasite, the differential expression of Chitotriosidase 1 homologs (Chit1) in mussels and cuticle proteins in *M. intestinalis* could be another example of a direct coupling of gene expression between hosts and parasites. As an essential component of the innate immunity against chitin-coated pathogens, Chit1 also regulates inflammatory processes (Elmonem *et al.* 2016; Van Eijk *et al.* 2005). However, due to its digestive function in mussels (Birkbeck & McHenry 1984; Lesser & Macmanes 2016), oysters (Yang *et al.* 2015) and insectivorous scorpions (Fuzita *et al.* 2015), it is also another example of the dual roles of genes in digestion and immunity. Interestingly, the upregulation of Chit1 corresponded to the upregulation of a number

of cuticle proteins in the parasites infecting Texel hosts (Figures 5.4, S5.1, and S5.2), suggesting that direct targeting of the parasite's chitin cuticle might have led to the parasite's response of strengthening the weakened exoskeleton with increased cuticle production. Since we only captured successful infections of parasites that were able to respond to the host attack, higher resistance in Texel mussels and higher infectivity of Texel parasites could be explained by successful expulsion of parasites that failed to counter the host chitinase immune effector by upregulation of their cuticle production. Hence, the coupling of host Chit1 and parasite cuticle proteins might characterise another direct interaction and help to explain how different evolutionary trajectories were realised along both invasion fronts (*Chapter 4, Feis et al. 2016*).

## **Conclusion**

In this study, our dual-species transcriptomics approach elucidated some molecular underpinnings of the young co-evolutionary relationship between mussels *M. edulis* and their intestinal macroparasites *M. intestinalis*. The mechanistic interaction pathways described by several candidate genes partly matched the different evolutionary trajectories of Mytilicola along the different invasion routes that have evolved over ~45 years since the introduction of the parasite, corresponding to roughly 45 host and probably around 100 parasite generations. Therefore, the observed changes occurred rapidly and had to rely either on a sufficiently large standing genetic variation of both host and parasite populations or on more plastic mechanisms like gene regulation. Similar to many other nonmodel organism transcriptome studies (e.g., Gallardo-Escárate *et al.* 2014; Patnaik *et al.* 2016; Qi *et al.* 2016; Yarra *et al.* 2016), a significant proportion of assembled contigs could not be functionally annotated and we are aware that we may have missed novel functional aspects of co-evolutionary interactions. Our results based on homology annotation revealed that the direct interaction between host and parasites can generate specificity of general responses (ROS pathways) on the transcription level within few generations, illustrating the value of invasive species for the study of host–parasite co-evolution in nature. Although RNA-seq is in principle a hypothesis-generating tool that cannot demonstrate causality, it can nevertheless be useful for identifying target genes and processes. Furthermore, transcriptional responses can help to characterise

the observed phenotypes and describe co-evolutionary patterns. Future studies should focus on the functional significance of differential gene regulation in this system and try to separate plasticity of the regulatory processes from adaptive evolution fixing underlying regulatory mutations. Here, we have mainly observed effects of *M. intestinalis* infestation and local adaptation on the effector side with our transcriptomics approach, but the underlying evolutionary changes, that is, the evolutionary changes at the DNA level that cause the patterns, are still elusive. Targeting the upstream steps of the involved pathways will therefore aid the search for key players in the co-evolutionary process in natural host–parasite systems.

## **Acknowledgements**

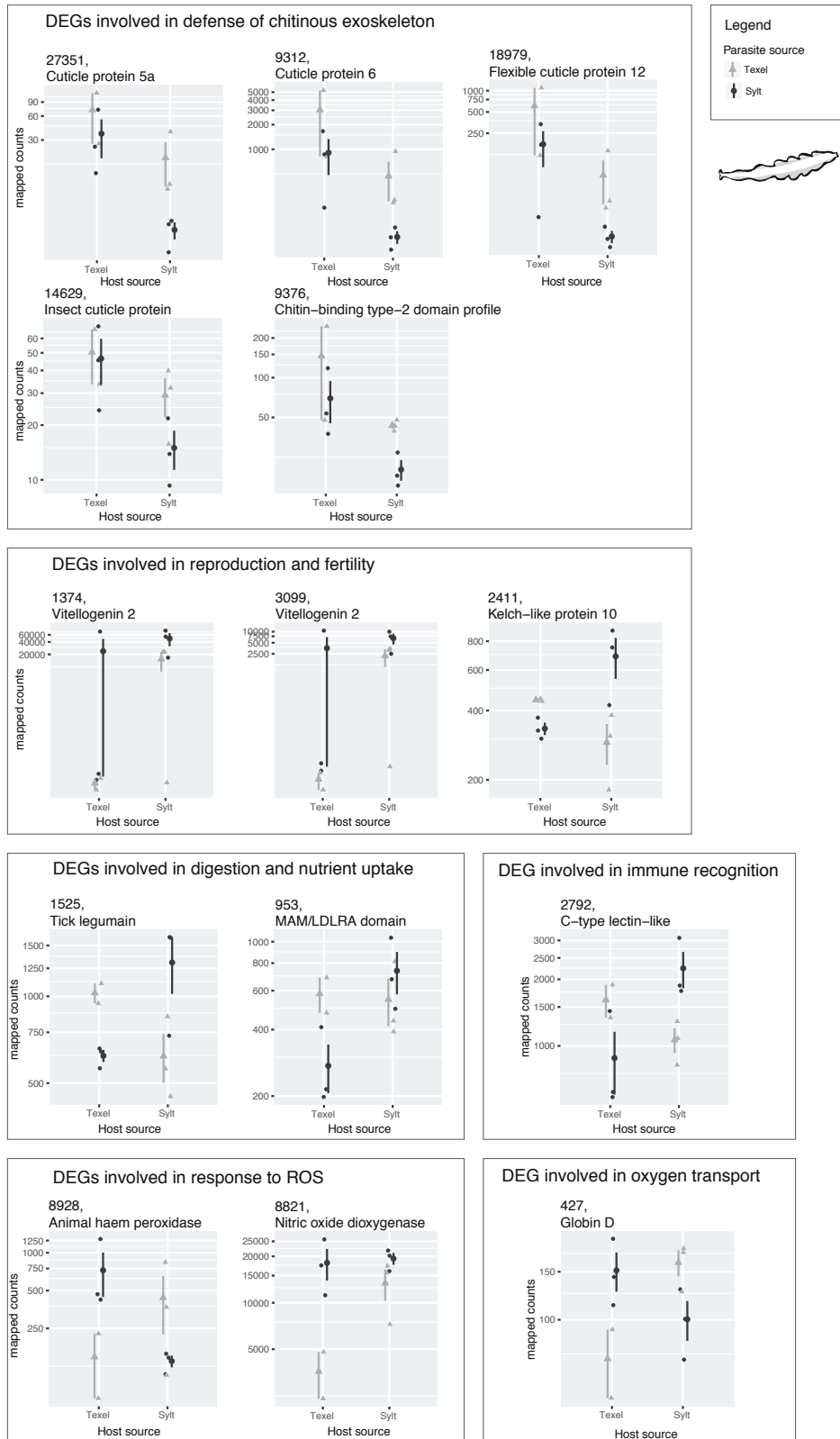
This work was supported by the German Research Foundation (DFG) Special Programme 1399 on host–parasite co-evolution (grant number We4641/1-2 to K.M.W.). The authors wish to thank David Thieltges and Anouk Goedknecht for their help in the field, Kaibil Escobar Wolf for his technical assistance in preparation and during the experiment, Nancy Kuehne for guidance with library preparation and running Illumina and Lars Harms for his support with bioinformatic work on the servers. We thank three anonymous reviewers and Camille Bonneaud for their comments, which greatly helped improve this manuscript.

## **Data accessibility**

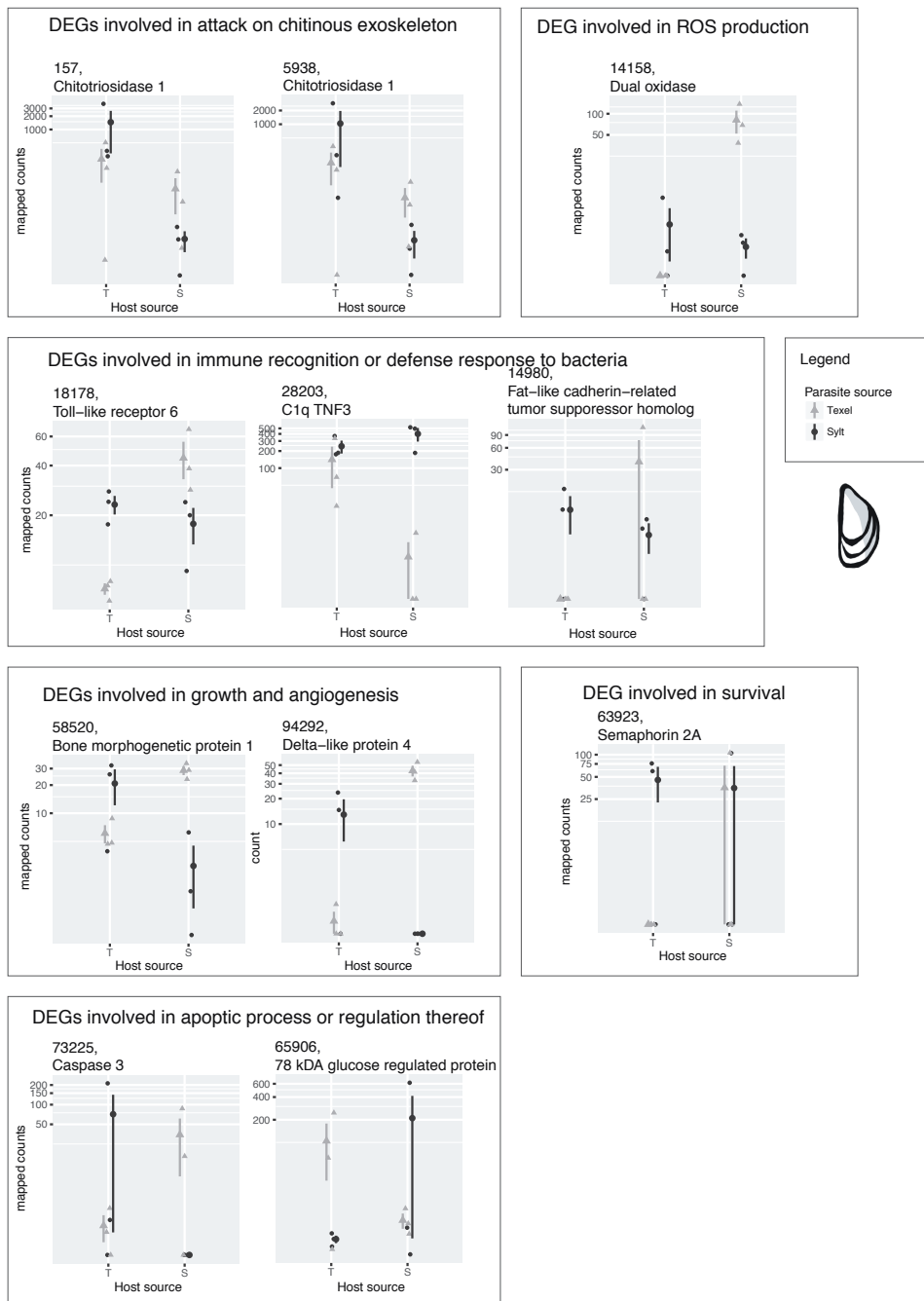
Raw RNA-seq data are available in the BioProject PRJNA430138 on NCBI with BioSample Accession numbers SAMN08394988 – SAMN08395065, which is linked to the Short Reads Archive study number SRP131446, Accession numbers SRR6513662 – SRR6513728. Mussel *M. edulis* and parasite *M. intestinalis* assemblies and corresponding annotation tables are available upon request.



## Supplementary materials



**Figure S5.1** *Mytilicola intestinalis* Differentially Expressed Genes (DEG): transcripts of homologs of four insect cuticle proteins and chitin-binding type-2, digestive proteins, haem-binding protein, ROS antagonists and proteins related to reproduction that are differentially expressed in the sympatry vs. allopatry contrast.



**Figure S5.2** *Mytilus edulis* Differentially Expressed Genes (DEG): transcripts of homologs of Chitotriosidase 1 and transcripts of contigs that were significantly expressed in the sympatry contrast (host source:parasite source) that were offspring of the GO processes response to stimulus (GO:0050896), immune system processes (GO:0002376) and death (GO:0016265).

**Table S5.1** BUSCO (Benchmarking Universal Single-Copy Orthologs; Simao *et al.* 2015) to explore completeness according to conserved ortholog content for *Mytilicola intestinalis*.

Eukaryota	C: 69% [D:n.a.], F: 7.7%, M: 14.2%, n:429
Metazoa	C: 68% [D:n.a.], F: 11.8%, M: 14.0%, n:843
Arthropoda	C: 51% [D:n.a.], F: 12.2%, M: 31.4%, n:2675

**Tables S5.2, S5.3** and **S5.4** of the Additional Supporting Information may be found online at Molecular Ecology in the supporting information tab for this article;

- **Table S5.2** is an annotation table including LFCs of all *Mytilus edulis* differentially expressed contigs in the control vs. infected dataset.
- **Table S5.3** is an annotation table including LFCs of all *Mytilus edulis* differentially expressed contigs in the sympatric vs. allopatric dataset.
- **Table S5.4** is an annotation table including LFCs of all *Mytilicola intestinalis* differentially expressed contigs.



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General discussion

**Marieke E. Feis**

## Synthesis

This dissertation addresses the study of host–parasite co-evolution and co-infection in the wild. The biological invasions of two parasitic copepods *Mytilicola intestinalis* and *Mytilicola orientalis* in the Wadden Sea were used as tools for this, because of the possibility to do carefully controlled laboratory experiments and because their invasion routes within the Wadden Sea have been well recorded, which made it possible to take hosts and parasites from locations with known similar and dissimilar amounts of co-evolutionary time. To address the aims in my thesis, I used a range of methods, which included extensive literature searches in combination with fieldwork and invasion genetics (*Chapter 2*), as well as laboratory experiments (*Chapters 3 and 4*) in combination with dual-species transcriptomics (*Chapter 5*).

To date, the invasions of the two marine parasites *M. intestinalis* and *M. orientalis* have been studied extensively because of their potential economic threats to the mussel and oyster aquaculture. *Mytilicola intestinalis* invaded the Atlantic and North Sea coasts of Europe through ship hull fouling and relaying of infected mussels (e.g., Caspers 1939; Ellenby 1947; Korrington 1968), whereas *M. orientalis* was co-introduced with the Pacific oyster *Magallana gigas* to the Pacific coast of North America (e.g., Odlaug 1946) and the Atlantic, North Sea and Mediterranean coasts of Europe (e.g., Clanzig 1989; Elsner *et al.* 2010; His 1977b; Stock 1993). However, validation of the anecdotal native ranges and invasion routes of both parasites were still lacking. I addressed this gap in knowledge in *Chapter 2*, in which I searched the available *Mytilicola* literature for recorded occurrences and absences throughout history. Against this backdrop, I could then formulate specific hypotheses about the invasions of both parasites, which I tested with invasion genetics of the mitochondrial COI gene of samples from the putative native regions and the invaded regions. Results from the invasion genetics were only partly consistent with the hypotheses. The invasion routes and native region of *M. orientalis* were consistent with the hypotheses: *M. orientalis* is native in Japan, where genetic diversity was high and population structure was found, and it is invasive in North America and Europe, where an equally high genetic diversity was found, but no population structure among samples and a lower structure overall. *Mytilicola orientalis* must have been co-imported in relatively large numbers, because no genetic diversity was lost upon co-introduction and invasion in the non-native ranges (i.e., no bottleneck). The

implication of this is that there is a substantial amount of standing genetic variation for selection to act upon. On the other hand, it was not possible to determine the native range of *M. intestinalis*, because of a lack of structure and a low genetic diversity in the putative native range, the Mediterranean Sea. However, it is clear from past studies that *M. intestinalis* is an invasive species in the Atlantic, as in many locations monitoring practices were in place which recorded an absence and later a presence of the parasite. Perhaps in the Mediterranean, *M. intestinalis* should be regarded as a cryptogenic species (a species whose origin cannot be readily determined on the available data, *sensu* Carlton 1996). The overall low genetic diversity in *M. intestinalis* implies there is less standing genetic variation for selection to act upon than in *M. orientalis*, which may have evolutionary consequences for their invasion trajectories (although in the eel–nematode system there was low variation in the invaded range and traits underlying host–parasite interactions could evolve anyway, Weclawski *et al.* 2014; Wielgoss *et al.* 2008). The invasion histories and demographies thus differ between *M. intestinalis* and *M. orientalis*, which may impact their further invasions and evolutionary trajectories, but also their competition in the invaded regions where they now co-occur.

The biological invasion of *M. intestinalis* and later *M. orientalis* created a situation in which two congeneric species co-occur and share the same host in their invaded range, in which they share a similar niche (Goedknecht *et al.* 2017b; Gresty & Quarmby 1991). The two species differ in their degree of host specificity, because *M. intestinalis* only infects the blue mussel *Mytilus edulis* and can thus be considered a specialist, while *M. orientalis* also infects Pacific oysters *M. gigas*, Baltic tellins *Limecola balthica* and common edible cockles *Cerastoderma edule* (Goedknecht *et al.* 2017a) and is therefore considered a generalist. However, generalists and specialists are not expected to occupy the same niche over extended periods of time, as specialist parasites should have a competitive advantage over generalists due to costs of generalism (Dall & Cuthill 1997; Futuyma & Moreno 1988; MacArthur 1972). Therefore in *Chapter 3* I performed experiments to investigate the competitive balance between *M. intestinalis* and *M. orientalis*, and to investigate what effect co-infections may have for the host condition. First, I showed that the Pacific oyster *M. gigas* is not a suitable host for *M. intestinalis*, which confirms the results of earlier field and lab experiments on Pacific oyster seed (Dare 1982) and which indicates that if *M. intestinalis* ends up in *M. gigas*, it is likely a dead end. Therefore, *M. gigas* may

act a diluter for *M. intestinalis*, while it acts as an amplifier for *M. orientalis*. Next, there were no indications of direct competition or superiority of either parasite in co-infections of mussels. The infection rate of *M. orientalis* was lower than that of *M. intestinalis*, which could be interpreted as a cost to its generalism. Apart from that, no cumulative effects of co-infections on host condition were found in this experiment, but an effect of dose due to the experimental design cannot be ruled out here. Therefore, ongoing invasion of *M. orientalis* in the northern Wadden Sea at locations where *M. intestinalis* is already present is more likely to be facilitated by the other hosts *M. orientalis* can utilise, than by competitiveness within the shared host.

Possibilities to perform time-shift experiments to study host–parasite co-evolution on natural systems are restricted to few systems, of which many rely on asexually reproducing hosts and parasites. In *Chapter 4*, I highlighted the use of parasite biological invasions to study co-evolution in nature, which can help reduce this bias towards microparasites. Again I used the biological invasion of *M. intestinalis* to study a phenomenon in the wild, but this time it was more focused on evolution. By making experimental cross-infections of hosts and parasites from two invasion fronts with similar co-evolutionary time (Texel and Sylt) and an uninvaded location (Kiel), I could study the effect of evolutionary time on host–parasite interactions (as a space-for-time substitution, Reusch 2014). Kiel mussels lacked a co-evolved response to parasite infection. Furthermore, invasion of *M. intestinalis* at separate fronts in the Wadden Sea led to different evolutionary trajectories on both host and parasite side in the mussel–*M. intestinalis* system, with pre-infection traits evolving on Texel, while post-infection traits may have evolved on Sylt. Therefore, the evolutionary outcomes may not always be predictable or may lead to the same pattern, and they may also depend on the biotic and abiotic environment. For instance, it might be possible that the presence of *M. orientalis* on Texel drove *M. intestinalis*' higher infectivity, while at the time of the experiments *M. orientalis* had not settled on the Sylt oyster reef.

The underlying molecular mechanisms and evolutionary steps of the antagonistic co-evolution between hosts and their parasites remain elusive, especially in non-model systems, because of the limited empirical support for the widespread phenomenon of host–parasite co-evolution. I addressed this gap of knowledge in *Chapter 5*, in which I used a dual-species transcriptomics approach on several experimental contrasts derived from the experiments in *Chapter 4* to document the



molecular underpinnings of the evolutionary trajectories. We extended a dual transcriptomics approach, in which host and (micro-)parasites are extracted and sequenced together and are later separated using bioinformatics, to a dual-species transcriptomics approach, in which host and parasite were extracted and sequenced separately. So far, dual transcriptomics studies had mainly focused on the infection process as such (e.g., Choi *et al.* 2014; Pittman *et al.* 2014; Rosani *et al.* 2015; Tierney *et al.* 2012), but had not incorporated the geographical aspects of co-evolutionary rapid local adaptation or contrasted sympatric and allopatric combinations of parasites. I demonstrated the immune response of mussels towards infections by *M. intestinalis* in general, which was mainly reflected in cell division processes, wound healing, apoptosis and the production of reactive oxygen species. Furthermore, by functionally coupling host and parasite sympatry contrasts, I could reveal several candidate genes that mediate the different evolutionary trajectories within the parasite's invasion. The host–parasite combination-specific coupling of effector mechanisms suggest that underlying recognition mechanisms create specificity and local adaptation. The combined approach of measuring phenotypic responses of hosts and parasites and performing dual-species transcriptomics thus promises to be very fruitful for unraveling the molecular mechanisms behind the evolution of host–parasite interactions in many other non-model species.

## **Future challenges**

### **On genetics and transcriptomics of *Mytilicola intestinalis***

*Mytilicola intestinalis* samples from Mediterranean and invaded locations, including Texel and Sylt, could not be separated into distinct populations, and there was very low overall genetic structure, as well as low nucleotide and haplotype genetic diversity (*Chapter 2*). Yet, the invasion of *M. intestinalis* towards separate ends of the Wadden Sea led to different evolutionary trajectories (*Chapter 4*), which have molecular underpinnings that were uncovered by dual-species transcriptomics (*Chapter 5*). These findings contrast each other: populations could not be differentiated based on their genetics, but co-evolution was a local process, with local differences in phenotype and transcription (which may be plastic, may have a genetic basis, or both). Probably our chosen marker for the invasion genetics does not offer

enough resolution on contemporary times. On the other hand, the majority of transcribed genes was not differentially expressed, and only a minority constituted to the local patterns found. Here, the existing RNA datasets could be of use to elucidate genetic differences between Texel and Sylt. Indeed, it would be interesting to study the DNA variation in both mussels and *M. intestinalis* to quantify the relative importance of plasticity and genetic determinism in the observed patterns of *Chapter 5*. In the existing RNA sequence data, SNP variants could be called to find evolutionary changes at the DNA level. Then, a calculation of  $F_{ST}$  between Texel and Sylt, as well as Tajima's D and nucleotide diversity could be used to test whether differentially expressed genes also display signatures of recent positive selection. This would give insights into the genetic basis of the mussel–*M. intestinalis* interactions, while at the same time, this could solve the apparent contrast between the conclusions of *Chapter 2* compared to those of *Chapter 4* and *5*. Furthermore, once positively selected and neutral SNPs are found, one could develop markers to add to the *M. intestinalis* phylogeographic analysis, which should give more resolution about the invasion routes, and at the same time, may resolve genetic structure in the Mediterranean basins and thus help elucidate whether the COI data mainly showed the signature of a selective sweep. Moreover, it should shed light on how local or how general the conclusions from *Chapters 4* and *5* are within the mussel–*M. intestinalis* system.

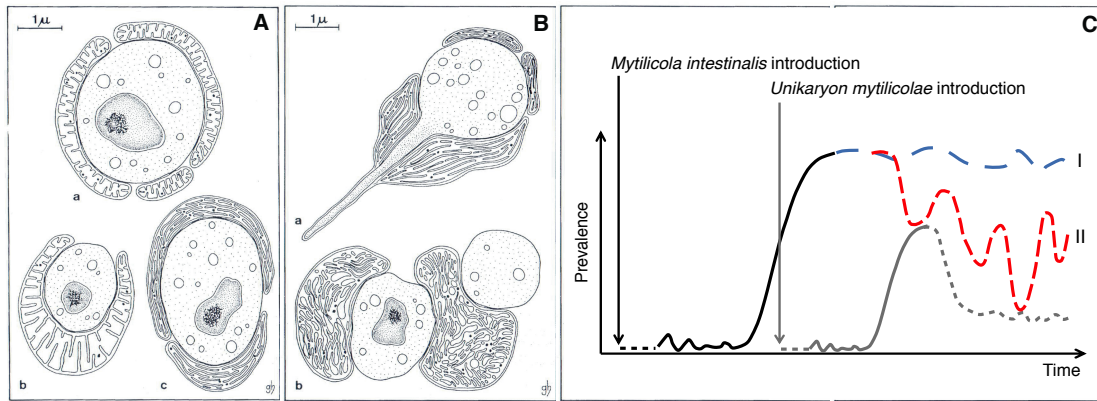
### **On the roles of the microbiome, defensive microbes and hyperparasites**

Increasingly, the role of the microbiome is being recognised as an important factor that could influence and mediate the antagonistic interactions between hosts and parasites. The ecological relatedness of host-associated microbial communities parallels the phylogeny of related host species (i.e., phylosymbiosis) (Brooks *et al.* 2016). It can be argued that hosts should not be viewed as autonomous entities, but should rather be viewed as “holobionts” that are composed of the host plus all of its symbiotic microbes (Bordenstein & Theis 2015). These symbiotic microbes have been shown to influence the susceptibility towards parasites (e.g., Ford & King 2016; King *et al.* 2016; Koch & Schmid-Hempel 2011; Mockler *et al.* 2018; Ritpitakphong *et al.* 2016). Ford *et al.* (2017) experimentally demonstrated co-evolutionary dynamics between a defensive microbe and a pathogen within a nematode host, which was driven by fluctuating selection dynamics. Thus defensive microbes could be

sources of selection on parasites, which drive fast evolutionary rates and divergent evolutionary trajectories (Ford *et al.* 2017).

The role of the microbiome is an aspect that I have not focused on in my experimental work on the mussel–*M. intestinalis* system. Indeed it has been an assumption that the microbial communities of Texel, Sylt and Kiel hosts are the same or influence the host–parasite traits in the same way. This may not be the case, and it is possible that the different evolutionary trajectories at the different parasite invasion fronts might be explained by differences in the microbial communities as one of the biotic factors mentioned in *Chapter 4*. Whether potential differences in microbiome are causes, consequences, or have nothing to do with differences in the mussel–*M. intestinalis* interactions should be elucidated with further experimental work in which this is manipulated and measured.

Another factor that could influence the mussel–*M. intestinalis* relationship is hyperparasitism by the microsporidian *Unikaryon mytilicolae* (Durfort *et al.* 1980). This hyperparasite is sexually transmitted between *M. intestinalis* (Durfort *et al.* 1985; Durfort & Vallmitjana 1982). Its presence in sperm, gametes and eggs does not make them unviable, and when *M. intestinalis* eggs hatch, the naupliar and copepodite stages will subsequently carry the microsporidian (Durfort *et al.* 1985). An association was found between the hyperparasite and the host mitochondria (Durfort & Vallmitjana 1982): *U. mytilicolae* trophozoites do not have mitochondria, and the mitochondria of the *M. intestinalis* cells are distributed around the trophozoites of *U. mytilicolae* (Figure 6.1A, B; Durfort *et al.* 1987). *Unikaryon mytilicolae* has, however, only been studied in *M. intestinalis* from the Galician rías in Spain, where it occurs in a mere 2% of the *M. intestinalis* population (Durfort *et al.* 1985; Durfort & Vallmitjana 1982). Whether it occurs elsewhere is unknown; none of the publications listed in the Supplementary material of *Chapter 2* mentions presence/absence of *U. mytilicolae* (apart from those cited in this paragraph). Did *U. mytilicolae* co-invade into the North Sea with *M. intestinalis*? Might the initial outbreaks of *M. intestinalis* in the invaded range be linked to “parasite–parasite release” of *U. mytilicolae* (Figure 6.1C)? Does *U. mytilicolae* influence the virulence of *M. intestinalis*, for instance through higher energy demand? Could it mediate the host–parasite interactions between mussels and *M. intestinalis*, and how would that influence host–parasite co-evolution?



**Figure 6.1** **A)** Schematic drawings of a) *Unikaryon mytilicolae* trophozoite surrounded by three elongated mitochondria; b) a mega-mitochondrion almost completely surrounds a trophozoite; c) the mitochondrial crests in this case adopt a disposition parallel to the major axis of the mitochondria. **B)** Schematic drawings; a) a mega-mitochondrion completely surrounds a surprising evagination that emits the trophozoite; b) observe the vesiculation of the mitochondrial crests. Figure 6.1A and B including caption (translated from Spanish) were taken from Durfort *et al.* (1987). **C)** Hypothetical invasion trajectories of *Mytilicola intestinalis* (black, blue and red) and *Unikaryon mytilicolae* (in grey). Establishment and expansion phase of *M. intestinalis* are indicated in black. The expansion phase would represent a “parasite–parasite release” effect. The *M. intestinalis* invasion scenario I (in blue), in which it keeps its dominance, is if *U. mytilicolae* did not invade. Alternatively, in scenario II (in red) the invasion of *U. mytilicolae* causes strong fluctuations of the *M. intestinalis* population. Representation of the hypothetical *U. mytilicolae* invasion presented here follows a boom–bust scenario with steady and low prevalence; this was chosen because of the very low prevalence in Galicia, which might reflect the long-term stable prevalence of *U. mytilicolae* in *M. intestinalis* populations; but of course the hypothetical *U. mytilicolae* invasion could follow a different scenario as well (similar to *M. intestinalis* invasion scenarios I or II). Figure 6.1C was redrawn after Reise *et al.* (2017a), with *U. mytilicolae* invasion added.

In a different light, perhaps the hyperparasite *U. mytilicolae* could be of use to solve the confusing puzzle of the native origin of its host *M. intestinalis* by comparing the phylogeography of the hyperparasite to that of the parasite. Other studies have used parasites as proxies to understand the genetic patterns of their host, and to solve cryptogenic origins of the host (Blakeslee *et al.* 2008), or to use them as biological tags (reviewed in Catalano *et al.* 2014). For this to work for *M. intestinalis*, the prevalence of the hyperparasite *U. mytilicolae* in *M. intestinalis* populations will be key – with 2% prevalence, obtaining enough material for a comparative parasite–hyperparasite phylogeographic study will take a lot of effort.

### On lessons to be drawn from a *Mytilicola* spp. literature review

Reviewing the extensive *Mytilicola intestinalis* and *Mytilicola orientalis* literature of course showed the advance of their respective invasions, but further clearly showed the waxing and waning of research interest in both species through time. Furthermore,

like with other invasive species (e.g., green crabs *Carcinus maenas* (Roman & Palumbi 2004), emerald ash borers *Agrilus planipennis* (Orlova-Bienkowskaja & Volkovitsh 2017) and the majority of studies on exotic plants (Hierro *et al.* 2005), *M. intestinalis* and especially *M. orientalis* have mainly been studied in the invaded range and rarely in the (perceived) native range. Orlova-Bienkowskaja and Volkovitsh (2017) further underline the importance of knowing the native range for invasive species: it is important for understanding their biology, for identifying potential natural enemies that may be used for biological control, and for ecological niche modelling to infer potential invasive distributions and impacts. To map the native and invaded ranges, I have noticed the importance of availability of old records in search engines and (online) libraries for the reconstruction of historical species distribution and invasion routes, and also the importance of publishing negative results in parasitology and being specific about those findings – for instance, absence in many mussel beds in the western Wadden Sea: which beds, how many beds, and how many samples were investigated per mussel bed? From the literature I collected to put together the invasion maps (*Chapter 2*), I have also recorded location, year, number of dissected hosts prevalence, and mean infection intensity (data not shown). These datasets could be analysed to calculate the speed of invasion of *M. intestinalis* and *M. orientalis*, and, furthermore, to calculate the approximate co-evolutionary time of each data point. Based on this, it would be interesting to compare parasite prevalence and intensity of both species in relation to the age of parasite–host combination, but also in relation to, for instance, latitude and distance to other locations where the parasite is present. Furthermore, which invasion scenario did *M. intestinalis* and *M. orientalis* follow; a scenario where dominance is maintained, a scenario with strong fluctuations caused by environmental, intrinsic and enemy effects, or perhaps a boom and bust scenario? And is this location-dependent? Furthermore, if prevalence and mean infection intensity correlate, does this correlation vary depending on the host–parasite combinations and on the invasion age? Can the epidemic phase be distinguished from the endemic phase for both species, and if so, how long did the epidemic phase last, and are there spatial differences?

Pacific oysters were reported from the Wadden Sea earlier than *M. orientalis* was (Elsner *et al.* 2010; Goedknecht 2017; Pogoda *et al.* 2012; Troost 2010). It would be interesting to find out how well the *M. orientalis* invasion matches the Pacific oyster invasion, and to calculate the lag and whether the abundance and suitability of

alternative hosts shortens invasion lag time. For this, a review of Pacific oyster presence/absence is necessary. Should lag differ between locations, these locations could be selected for experiments to test whether the lag of invasion matters for host and parasite infection traits but also dispersal traits in this system, similar to the cane toad studies in Australia, where parasites at the range-edge matured faster and produced small numbers of large eggs as opposed to parasites from the population core (Kelehear *et al.* 2012; Phillips *et al.* 2010).

### **Concluding remarks**

In this dissertation, I have shown the value of using biological parasite invasions to study ecological and evolutionary processes in nature. The natural histories of the invasive parasitic copepods of the *Mytilicola* genus, as well as the possibility to take them into the lab and perform experiments made them ideal candidates to study co-infection and co-evolution in nature. The use of dual-species transcriptomics on sympatric and allopatric host–parasite combinations and a control group was novel, and yielded co-evolutionary mechanisms between mussels and *M. intestinalis* based on several candidate processes and genes. Future work within this system should focus on quantifying the relative importance of plasticity and genetic determinism of these interactions.

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Summary

Zusammenfassung

## Summary

Parasites are ubiquitous in nature and the strong natural selection pressures they impose on their hosts are predicted to elicit rapid evolutionary adaptive responses of hosts towards their parasites, leading to antagonistic host–parasite co-evolution. While most of what we know about these processes stems from laboratory studies with a restricted set of model organisms, much less is known about host–parasite co-evolution in the wild, largely due to logistical challenges of experimental manipulations on an ecosystem scale. Biological invasions represent excellent opportunities to overcome these logistical issues and study host–parasite interactions and co-evolution in non-model species in the wild, as they can be seen as “natural experiments” in which different invasion scenarios lead to different ecological and evolutionary dynamics. This dissertation utilises the invasions of two parasitic copepods *Mytilicola intestinalis* and *Mytilicola orientalis* in the Wadden Sea to address the study of host–parasite co-evolution and co-infection in non-model species in the wild. This conceptual approach and the biological system are introduced in *Chapter 1*.

Validation of the presumed origins and invasion routes of both parasites were still lacking, therefore my first aim was to confirm them. This was done through an extensive literature review, in combination with invasion genetics (*Chapter 2*), which resulted in the validation of Japan as the native and North America and Europe as the invaded ranges of *M. orientalis* (where it was co-introduced with the Pacific oyster *Magallana gigas*), while it remained unclear whether the Mediterranean Sea is really the native range of *M. intestinalis*.

The biological invasion of *M. intestinalis* and later *M. orientalis* created a situation in which two congeneric species co-occur and share the same novel host in their invaded range. The two parasites have different degrees of host specificity in the Wadden Sea, as *M. intestinalis* only infects blue mussels *Mytilus edulis*, while *M. orientalis* also infects Pacific oysters *M. gigas*, and at least two other bivalve species. As generalists and specialists are not expected to occupy the same niche over extended periods of time, my second aim was to investigate potential competitive interactions between the two parasites. In an experiment, I showed that Pacific oysters are not suitable hosts for *M. intestinalis* (*Chapter 3*). Furthermore, from an infection experiment with mussels, there were no indications of direct competition or

superiority of either parasite in co-infections, but the infection rate of *M. orientalis* was lower than that of *M. intestinalis* (Chapter 3). Therefore, the ongoing invasion of *M. orientalis* in the Northern Wadden Sea at locations where *M. intestinalis* is already present is more likely to be facilitated by the other hosts *M. orientalis* can utilise, than by competitive interactions within the shared host.

Moreover, I highlighted the use of biological invasions of parasites to study host–parasite co-evolution in non-model organisms in the wild. By making experimental cross-infections of mussels and *M. intestinalis* from two invasion fronts with similar co-evolutionary time periods (Texel and Sylt) and an uninvaded location (Kiel), I could study evolutionary changes after introduction. Kiel mussels lacked a co-evolved response to parasite infection. Furthermore, invasions of *M. intestinalis* at separate fronts in the Wadden Sea led to different evolutionary trajectories in both hosts and parasites in the mussel–*M. intestinalis* system, with pre-infection traits (infectivity/resistance) evolving on Texel, while post-infection traits (virulence/tolerance) may have evolved on Sylt (Chapter 4). A dual-species transcriptomics approach on Texel and Sylt hosts and parasites of the sympatric, allopatric and control treatments was used next to document the molecular underpinnings of the different evolutionary trajectories. This uncovered a general immune response of mussels towards *M. intestinalis* infections, as well as several candidate genes and processes that mediate the different evolutionary trajectories (Chapter 5). The combination of studying responses on the phenotypic level and the transcriptional level through dual transcriptomics thus promises to be a fruitful approach to unravel the molecular mechanisms behind evolution of host–parasite interactions in many other non-model organisms.

In general, this dissertation demonstrates the value of using biological invasions to investigate host–parasite co-evolution in the wild, which is especially promising for research on non-model organisms. Future research should, however, further unravel the relative importance of phenotypic plasticity and genetic determinism for the co-evolutionary dynamics between hosts and parasites, but should also investigate the potential effects of other factors, such as, in the case of *Mytilicola*, the roles of the mussel microbiome and the hyperparasite *Unikaryon mytilicolae* (discussed in Chapter 6).

## Zusammenfassung

Parasiten sind in der Natur allgegenwärtig und es wird angenommen, dass der starke Selektionsdruck, den sie auf ihre Wirte ausüben, zu zu schnellen evolutionären Anpassungen der Wirte gegenüber ihren Parasiten führt und damit zu einer antagonistischen Koevolution von Wirt und Parasit. Unser Wissen über diese Prozesse stammt vor allem aus Laboruntersuchungen mit einer beschränkten Anzahl von Modellorganismen. Weit weniger untersucht ist hingegen die Koevolution von Wirten und Parasiten in natürlichen Systemen, vor allem aufgrund der logistischen Herausforderung experimenteller Manipulationen auf Ökosystemebene. Biologische Invasionen bieten ausgezeichnete Möglichkeiten, diese Komplikationen zu umgehen, und Wirt-Parasit-Interaktionen und Koevolution von Nichtmodellorganismen im Freiland zu untersuchen, da sie als "natürliche Experimente" angesehen werden können, in welchen verschiedene Invasionsszenarien zu unterschiedlichen ökologischen und evolutionären Dynamiken führen. Diese Dissertation nutzt die Invasionen der beiden parasitären Ruderfußkrebse *Mytilicola intestinalis* und *Mytilicola orientalis* im Wattenmeer, um die Koevolution von Wirten und Parasiten sowie von Koinfektion in natürlichen System zu untersuchen. Dieser konzeptionelle Ansatz sowie das biologische System werden in Kapitel 1 vorgestellt.

Bisher gab es keine validierten Informationen über die Herkunft und Invasionsrouten beider Parasitenarten. Daher war es mein erstes Ziel, diese Wissenslücke zu füllen. Durch eine umfangreiche Literaturrecherche in Kombination mit Invasionsgenetik (Kapitel 2) konnte ich Japan als das ursprüngliche Verbreitungsgebiet validieren und demonstrieren, dass *M. orientalis* erst später in Nordamerika und Europa mit der Pazifischen Auster *Magallana gigas* eingeführt wurde. Unklar blieb jedoch, ob das Mittelmeer tatsächlich das ursprüngliche Verbreitungsgebiet von *M. intestinalis* ist.

Die biologische Invasion von *M. intestinalis*, und später die von *M. orientalis*, schuf eine Situation, in der zwei nah verwandte Arten zusammen vorkommen und sich den gleichen Wirt in ihrem neuen Verbreitungsgebiet teilen. Allerdings haben die beiden Parasiten im Wattenmeer unterschiedliche Wirtsspezifitäten. *M. intestinalis* infiziert nur Miesmuscheln *Mytilus edulis*, während *M. orientalis* auch Pazifische Austern *M. gigas* und mindestens zwei weitere Muschelarten infiziert. Generell ist zu erwarten, dass Generalisten und Spezialisten nicht über längere Zeiträume dieselbe

Nische besetzen können. Es war daher mein zweites Ziel, eine mögliche Konkurrenz zwischen den beiden Parasitenarten zu untersuchen. In einem ersten Experiment habe ich gezeigt, dass Pazifische Austern kein geeigneter Wirt für *M. intestinalis* sind (Kapitel 3). Mein Infektionsexperiment mit Miesmuscheln erbrachte jedoch keine Hinweise auf direkte Konkurrenz oder Überlegenheit einer der beiden Parasiten bei Koinfektionen. Allerdings war die Infektionsrate von *M. orientalis* geringer als die von *M. intestinalis* (Kapitel 3). Es ist daher wahrscheinlich, dass die fortschreitende Invasion von *M. orientalis* im nördlichen Wattenmeer, an Stellen an denen *M. intestinalis* bereits vorhanden ist, vor allem durch andere von *M. orientalis* genutzte Wirte bestimmt wird und nicht durch Konkurrenz innerhalb des gemeinsamen Wirts.

Des Weiteren hebe ich in dieser Dissertation hervor, wie biologische Invasionen zur Untersuchung von Koevolution von Wirten und Parasiten in Nichtmodellorganismen in freier Wildbahn genutzt werden können. Durch experimentelle Kreuzinfektionen von Miesmuscheln und *M. intestinalis* von zwei Invasionsfronten mit ähnlichen Koevolutionszeiträumen (Texel und Sylt) und von einem bisher nicht besiedelten Gebiet (Kiel) konnte ich evolutionäre Veränderungen seit der Erstansiedlung untersuchen. Kieler Miesmuscheln fehlte eine koevolutionäre Reaktion auf Parasiteninfektionen. Darüber hinaus führte die Invasion von *M. intestinalis* an den verschiedenen Fronten im Wattenmeer zu unterschiedlichen evolutionären Verläufen sowohl auf der Wirts- als auch auf der Parasitenseite. Während sich auf Texel Eigenschaften entwickelt haben, welche die Wirt-Parasit-Interaktion vor der Infektion beeinflussen (Infektiosität / Resistenz), sind auf Sylt wahrscheinlich Eigenschaften entstanden, die nach der Infektion die Wirt-Parasit-Interaktion beeinflussen (Virulenz / Toleranz) (Kapitel 4). Um die molekularen Grundlagen dieser verschiedenen evolutionären Verläufe zu dokumentieren, habe ich einen Zwei-Arten-Transkriptomik-Ansatz verwendet. Dabei wurden Wirte und Parasiten von Texel und Sylt aus den sympatrischen, allopatrischen und Kontrollbehandlungen verwendet. Die Analysen zeigten eine allgemeine Immunantwort von Miesmuscheln auf Infektionen mit *M. intestinalis* sowie mehrere Kandidatengene und Prozesse, welche den verschiedenen evolutionären Verläufen zugrunde liegen (Kapitel 5). Die Kombination von Untersuchungen auf der phänotypischen und der transkriptionellen Ebene durch duale Transkriptomik ist somit ein vielversprechender Ansatz, um die molekularen Mechanismen der

evolutiven Dynamik von Wirt–Parasit-Wechselwirkungen in anderen Arten als den üblichen Modellorganismen zu entschlüsseln.

Insgesamt demonstriert diese Dissertation die Nützlichkeit von biologischen Invasionen als natürliche Experimente, um die Koevolution von Wirten und Parasiten in natürlichen Systemen zu untersuchen, was vor allem im Hinblick auf Nichtmodellorganismen vielversprechend ist. Zukünftige Herausforderungen bei solchen Untersuchungen sind nicht nur die Quantifizierung der relativen Bedeutung von phänotypischer Plastizität und genetischem Determinismus für die koevolutionäre Dynamik zwischen Wirten und Parasiten, sondern auch der mögliche Einfluss anderer Faktoren, wie im Fall von *Mytilicola* die Rolle des Mikrobioms des Wirtes und des Hyperparasiten *Unikaryon mytilicolae* (Kapitel 6).



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## About the author

Marieke Eveline Feis was born on 29 August 1989 in 's-Gravenhage, the Netherlands. Her father, who taught biology at a high school and who loved explaining all things nature, opened her mind to the natural world. The decision what to study was therefore not difficult, and she subsequently moved to Groningen to study biology at the University of Groningen with a major in Marine Biology and a minor in Arctic and Antarctic Studies. After finishing her bachelor degree in 2010, she continued with the master Marine Biology at the same university. Her first research project on the dispersal of marine trematodes at the Royal Netherlands Institute for Sea Research was supervised by Dr. Pieterella Luttikhuisen and Dr. David Thieltges. The international collaboration within this project meant short stays in France, Denmark, Ireland and Morocco for parasite collection. The successful completion of the project furthermore led to her first peer-reviewed publication. After that, she did her second research project abroad with Marco Polo funding, to study climate change-induced evolution in mummichogs, which she did under the supervision of Dr. Erik Sotka and Prof. Dr. Allan Strand at the College of Charleston and Grice Marine Lab. These research projects were so far mainly lab-oriented, and she also wanted to do a project that had a heavier focus on fieldwork and ecology. She found an opportunity to do a 3.5-month research project led by PhD student Thomas Oudman (now Dr.), which included 2 months of fieldwork in Mauritania. In this project, she looked at the available food resources during winter for red knots on the tidal flats in the Parc Nationale du Banc d'Arguin. She graduated as a marine biologist in 2013 and started as a PhD candidate a month later in Dr. Mathias Wegner's group at the Alfred Wegener Institute in Germany. In 2018 she will defend her thesis entitled "*Host-parasite co-evolution and co-infection of invasive parasitic Mytilicola copepods in blue mussel hosts*". She is looking forward to her next step in science!

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## List of publications

### Peer reviewed publications

**Feis M. E.**, John U., Lokmer A., Luttikhuizen P. C., Wegner K. M. (2018) Dual transcriptomics reveals co-evolutionary mechanisms of intestinal parasite infections in blue mussels *Mytilus edulis*. *Molecular Ecology* 27: 1505-1519.

**Feis M. E.**, Goedknecht M. A., Thieltges D. W., Buschbaum C., Wegner K. M. (2016) Biological invasions and host–parasite co-evolution: different coevolutionary trajectories along separate parasite invasion fronts. *Zoology* 119: 366-374.

Goedknecht M. A., **Feis M. E.**, Wegner K. M., Luttikhuizen P. C., Buschbaum C., Camphuysen K. (C. J.), van der Meer J., Thieltges D. W. (2016) Parasites and marine invasions: Ecological and evolutionary perspectives. *Journal of Sea Research* 113: 11-27.

**Feis M. E.**, Thieltges D. W., Olsen J. L., de Montaudouin X., Jensen K. T., Bazairi H., Culloty S. C., Luttikhuizen P. C. (2015) The most vagile host as the main determinant of population connectivity in marine macroparasites. *Marine Ecology Progress Series* 520: 85-99.

### Submitted manuscripts

Oudman T., Ahmedou Salem M. V., **Feis M. E.**, Dekinga A., Holthuijsen S., van Gils J. A., Piersma T., Bijleveld A. I. From grouping nomads to solitary residents in one species: the resource environment sets the compromise between aggregation and site fidelity in red knots.

### Manuscripts in preparation

**Feis M. E.**, Goedknecht M. A., Arzul I., Chenuil A., den Boon O., Gottschalck L., Kondo Y., Ohtsuka S., Shama L. N. S., Thieltges D. W., Wegner K. M., Luttikhuizen P. C. Global invasion genetics of two parasitic copepods infecting marine bivalves.

**Feis M. E.**, Wegner K. M. Do invasions create competitors? Co-infections of specialist *Mytilicola intestinalis* vs. generalist *Mytilicola orientalis* in blue mussels.

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## Affidavit

I hereby confirm that the content and design of my thesis entitled:

**“Host–parasite co-evolution and co-infection of invasive parasitic *Mytilicola* copepods in blue mussel hosts”**

are the result of my own work apart from my supervisor’s guidance and co-operation with my co-authors. I prepared this thesis following the Rules of Good Scientific Practise of the German Research Foundation.

Furthermore, I confirm that this thesis has not been submitted as a part of another examination process neither in identical nor in similar form.

Parts of this thesis have been published or submitted to scientific journals:

- Chapter 3 has been published as: Feis M. E., Goedknecht M. A., Thieltges D. W., Buschbaum C., Wegner K. M. (2016) Biological invasions and host–parasite co-evolution: different coevolutionary trajectories along separate parasite invasion fronts. *Zoology* **119**, 366-374
- Chapter 4 has been published as: Feis M. E., John U., Lokmer A., Luttikhuizen P. C., Wegner K. M. (2018) Dual transcriptomics reveals co-evolutionary mechanisms of intestinal parasite infections in blue mussels *Mytilus edulis*. *Molecular Ecology* **27**, 1505-1519
- Chapter 1 is in preparation for submission with M. A. Goedknecht, I. Arzul, A. Chenuil, O. den Boon, L. Gottschalck, Y. Kondo, S. Ohtsuka, L. N. S. Shama, D. W. Thieltges, K. M. Wegner and P. C. Luttikhuizen
- Chapter 2 is in preparation for submission with K. M. Wegner

Kiel, May 2018

Marieke Eveline Feis