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Impact of the invasive parasitic copepod *Mytilicola orientalis* on native blue mussels *Mytilus edulis*

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Abstract

Invasive species can indirectly affect native species by modifying parasite-host dynamics and disease occurrence. This scenario applies to European coastal waters where the invasive Pacific oyster (*Crassostrea gigas*) co-introduced the parasitic copepod *Mytilicola orientalis* that spills over to native blue mussels (*Mytilus edulis*). In this study, we investigated for the first time the impact of *M. orientalis* infections on blue mussels by conducting a laboratory experiment using controlled infections with larval stages of the parasitic copepod. As the impact of infections is likely to depend on the mussels' food availability, we also tested whether potential adverse effects of infection on mussels intensify under low food conditions. Blue mussels that were experimentally infected with juvenile *M. orientalis* had significantly lower condition (11 - 13%) than uninfected mussels after nine weeks of infection. Likewise, naturally infected mussels from a mixed mussel and oyster bed had about 20% lower condition than uninfected mussels, however this difference was only marginally significant. Contrary to effects on mussel condition, we did not find an effect of experimental infections on clearance rates, growth or survival of blue mussels and no clear sign of exacerbating effects of food limitation. Our study illustrates that invasive species can indirectly affect native species via parasite co-introductions and parasite spillover and calls for the integration of such indirect effects of invasions in impact assessments of invasive species.

Introduction

Invasive species affect native species, communities and ecosystems worldwide (Davies, 2009; McGeoch et al., 2010; Lockwood et al., 2013), often directly via predation and competition (Parker et al., 1999; Simberloff et al., 2013). However, invasive species can also exert indirect effects, for example by changing habitat structure or modifying parasite-host dynamics. Parasite-mediated indirect effects of invasive species can take place via several mechanisms (reviewed in Goedknegt et al., 2016). One of these mechanisms is the co-introduction of a parasite with an invasive host species (*parasite co-introduction*; Lymbery et al., 2014). Consequently, in the invaded range, this co-introduced parasite might spill over from its invasive host to novel native host species (*parasite spillover*; Prenter et al., 2004; Kelly et al., 2009). This mechanism is common in marine ecosystems, where 73% of the known parasite co-introductions are followed by parasite spillover from invasive to native host species (Goedknegt et al., 2016). Almost half of these spillover events have led to emerging diseases and subsequent mass mortalities of native hosts with, in several cases, knock-on effects on native communities and ecosystems (reviewed by Goedknegt et al., 2016).

Parasite co-introduction and spillover have also occurred in the course of the introduction of the Pacific oyster (*Crassostrea gigas*; Thunberg, 1793) to European coastal waters for aquaculture purposes (Troost, 2010). With the initial oyster imports in the 1960s and 70s, the intestinal parasitic copepod *Mytilicola orientalis* (Mori, 1935) was co-introduced to Europe (His, 1977). The parasite's native range is in Japanese waters, and it has a direct life cycle with a free-living larval dispersal stage, after which it resides in the intestines of molluscs (Mori, 1935). After its co-introduction to Europe, the parasite spread first via its principle host, the Pacific oyster, but was later additionally found in various native mollusc species, indicating spillover events (His, 1977; Stock, 1993; Pogoda et al., 2012; Goedknegt et al., 2017). In particular, native blue mussels (*Mytilus edulis*; Linnaeus, 1758) are increasingly serving as new hosts, with infection prevalences being similar to or even exceeding those in Pacific oysters in some areas (Pogoda et al., 2012; Goedknegt et al., 2017). Native blue mussels are also infected by *Mytilicola intestinalis* (Steuer, 1902), a related parasite species introduced from to the Mediterranean Sea about 80 years ago, (Steuer, 1902; Caspers, 1939; Elsner et al., 2011; Goedknegt et al., 2017) which has a similar direct life cycle as *M. orientalis* (Caspers, 1939; Grainger, 1951; Dethlefsen, 1985; Gee and Davey, 1986a). *Mytilicola intestinalis* became infamous as the 'red worm disease', because it was blamed to be the causative agent of mass mortalities of blue mussels in the North Sea in the 1950s and 60s (Korringa, 1968; Blateau et al., 1992), however so far experimental evidence for negative impacts based on controlled infections of mussels is lacking. Especially juvenile stages of *M. intestinalis* were held responsible for mortalities of mussels (Korringa, 1950; Dethlefsen, 1985) due to their presence in the ramifications of the digestive gland (Campbell, 1970). In addition, the energy demand of young infective stages is expected to be high after exploiting the egg yolk and the lack of external feeding in the pelagic larval phase (based on observations of *M. intestinalis*; Grainger, 1951), thereby increasing the chance for mussels to become negatively affected by juvenile stage of the parasite. For mature *M. intestinalis*, lethal and sublethal effects on important host fitness components such as condition, filtration rate and reproduction do not seem to be one-directional and have been controversially discussed (e.g. review by Lauckner, 1983). Similarly, while the new invasive parasite *M. orientalis* is generally considered a serious pest (Holmes and Minchin, 1995) and registered in the list of 100 worst invaders of the Mediterranean (Streftaris and Zenetos, 2006), studies on the lethal and sublethal effects of *M. orientalis* are inconclusive for oyster hosts (*C. gigas*: Katkansky et al., 1967; Deslous-Paoli, 1981; De Grave et al., 1995; Steele

and Mulcahy, 2001; *Ostrea lurida:* Odlaug, 1946) and, like for *M. intestinalis*, they generally lack experimental approaches. Effects of *M. orientalis* on its new native host, the blue mussel, have not been studied to date. The impact of the invasive *M. orientalis* on native blue mussels is likely to be modified by environmental factors and species interactions (e.g. Campbell, 1970; Lauckner, 1983; Hepper, 1953; Troost, 2010). For example, seasonal cycles, extreme temperatures and inter- and intraspecific competition may lead to food limiting conditions that can either alleviate or intensify adverse effects of infection by the parasitic copepod. Along these lines, Moore et al. (1977) postulated that *M. intestinalis* actually lives in a commensal relationship with their host, but that this relationship can turn into a negative interaction in times of serious food limitation. However, experimental evidence for an augmented impact of *Mytilicola* spp. infections on hosts at low food levels is lacking.

In this study, we investigated for the first time the effects of *M. orientalis* infection on the native blue mussel *M. edulis* by conducting a laboratory experiment with controlled infections of mussels with larval stages of the copepods. Following the hypothesis of Moore et al. (1977) we also tested whether potential adverse effects of infection on survival, clearance rate, body condition and growth of blue mussels intensify with low food conditions. Next to this laboratory experiment, we collected mussels in the field to determine natural infection levels and to investigate relationships between *Mytilicola* infections and mussel condition. This combination of lab and field investigations allowed us to assess the impact of the spill over of the invasive parasite *M. orientalis* from invasive Pacific oysters on native blue mussels.

Material and methods

Field survey

To determine natural infection levels of *Mytilicola orientalis* in blue mussels (*Mytilus edulis*) of the size class used in the experiments, we collected 10 mussels of 30-35 mm length from a mixed (Pacific oysters (*Crassostrea gigas*) and blue mussels) bed located on the Vlakte van Kerken, a tidal flat on the east coast of the island of Texel (53°06'83.1" N; 4°55'19.8" E, Wadden Sea, The Netherlands) on 25 June 2014. Additionally, we collected another 39 mussels (34.1-53.6 mm) to analyse for the relationship between infection status (infected/uninfected) and mussel body condition. Before screening, we measured the length of each mussel shell to the nearest mm with callipers and then separated both shells. For methods on the assessments of infection status and condition index see section *Measurement of clearance rate, body condition and growth*.

Experimental infections

Uninfected mussels (30-35 mm) for the experiments were collected from basalt groins on the north-west shore of the Dutch mainland (Julianadorp, 52°52'55.8" N; 4°42'25.9" E). Previous explorations had shown that *Mytilicola* spp. do not occur at this location, which was verified by dissecting 30 mussels in which no infections were found. Any epifauna (mostly barnacles) on the mussels was carefully removed from the shells to ensure that copepod larvae could infect mussels without being predated or physically obstructed during experimental infections (Johnson and Thieltges, 2010). Until the infection procedure, collected mussels were maintained in 75 L flow-through tanks at 18°C under a 24-hour light cycle (12 h light and 12 h dark) and fed three times

per week with fresh *Isochrysis galbana* culture, or alternatively with Phyto-Feast® when fresh culture was unavailable.

To acquire a source for *M. orientalis* larvae, mussels were collected from a mixed bed with known *Mytilicola* infections located on a tidal flat on the east coast of the island of Texel (53°06'83.1" N, 4°55'19.8" E, Vlakte van Kerken, Wadden Sea, The Netherlands). Within two days of collection, mussels were dissected and *M. orientalis* gravid females extracted, which were identified and distinguished from *M. intestinalis* based on descriptions of Mori (1935) and Elsner et al. (2011). The egg sacs were separated from the female and placed in individual petri dishes (Ø 60 mm) filled with sea water. They were incubated at approximately 30°C to expedite the larval development time (based on results of a pilot study) and were monitored daily. Larval stages were identified based on descriptions of *M. intestinalis* larvae by Gee and Davey (1986a). When larvae had developed into the copepod I stage (infestive stage; Gee and Davey 1986a), uninfected mussels were challenged with the larvae. However, due to variation in larval hatching and development time, insufficient larvae were available to infect all mussels in one batch, ultimately resulting in two temporal batches of infected mussels, which formed the base of the experiment.

Before exposure to infestive larvae, the shell length of each mussel was measured with callipers to the nearest mm. As this was the first time that artificial infections were conducted with *M. orientalis*, we experimented with two infection protocols (Table 7.1). Individual mussels in the first batch of the experiment were exposed to parasites in a 100 mL cup and after 24 h mussels and sea water were transferred to a 1000 mL container for the following eight weeks of the experiment. For individual mussels in the second batch, exposure was carried out directly in the 1000 mL container, where they remained for the entire nine weeks of this study.

Exposure of mussels to infestive larvae was done by carefully pipetting (200 µl pipette) 25 larvae from the petri dish (by the use of a stereo microscope) and depositing them into the container with an uninfected mussel and sea water. To promote filtration and uptake of infestive larvae, small amounts of algal culture (*I. galbana*) were added to the sea water during exposure of mussels from batch two of the experiment. Five extra mussels per each of the two batches were infected and sacrificed for examination of larval development at mid-way points during the experiments. Control mussels were treated identically to exposed mussels within each batch (transfer of sea water) but without the addition of copepodites.

Experimental set-up

The experiment was set up immediately after exposing the mussels to the parasite larvae. The experiment was run in a two-factorial design with *M. orientalis* infection (infected/uninfected) and food level (high/low) as fixed factors and set up in blocks of four 1000 mL containers with a replicate of each of the four treatments. The containers were kept in a climate-controlled room at 18°C and sea water was replaced weekly. All mussels were fed three times per week with the same amount of *Isochrysis* algae mixture, with mussels in the high food treatment receiving 50 mL algae mixture and mussels in the low food treatment receiving half that quantity. When fresh algae culture was unavailable, 0.1 mL of PhytoFeast® per mussel was provided in the high food treatment, while 0.05 mL was provided to mussels in the low food treatment.

The first batch of the experiment (17 blocks) was commenced about three weeks earlier than the second batch (15 blocks) as determined by the availability of infective copepodite stages (Table 7.1). Survival of mussels was monitored daily, but no mortality was observed throughout the experiment. We conducted the first clearance rate measurements (see section *Measurement*

of clearance rate, body condition and growth for details) of the first batch of mussels approximately one week after exposure to *M. orientalis* larvae, while the clearance rate of the second batch of mussels was measured immediately (one day) after exposure. We continued to measure the clearance rate of each mussel once per week, to assess if and when larval maturation affected the clearance rate of mussels. The experiment ran for a maximum of nine weeks, after which all mussels were measured, screened for presence of *M. orientalis* larvae, frozen, freeze-dried and weighed to assess condition index (see section *Measurement of clearance rate, body condition and growth* for details).

Measurement of clearance rate, body condition and growth

Clearance rate

Clearance rate was assessed by means of the indirect clearance method (Riisgård, 2001; Petersen et al., 2004). One day prior to the measurement, seawater was refreshed in all containers. In the morning before the test, we made dilutions of live algal (*I. galbana*) culture and analysed its density using a CASY® Cell Counter and Analyser System Model TT (Schärfe System GmbH). The algal dilutions were calibrated to RFU (Relative Fluorescence Units) using a Trilogy® Laboratory Fluorometer (Turner Designs). During the test, algal concentration measurements were performed on the fluorometer as its high measurement rate allowed us to measure a large number of samples in a short period of time. Fluorometer measurements required 1.5 mL of water that we obtained from each experimental container with a 2 mL pipette.

We measured background fluorescence levels (RFU) immediately before the test and used a calibration curve to calculate the amount of algal culture needed to create an initial starting concentration of $13 \times 10^3 - 14 \times 10^3$ algal cells per mL in each experimental container. This level was chosen to avoid very high or very low algal densities which are known to hamper filtration of mussels (Clausen and Riisgård, 1996) and because it falls in the middle of the range in which mussel filtration rate is independent of food density (Riisgård and Randløv, 1981). Algal culture of the calculated quantity was added once to all experimental containers and fluorescence was measured immediately after addition (t_0) and again after one (t_1) and two hours (t_2). The measured RFU values at these measurement intervals were corrected for background fluorescence after which these values were transformed to number of algal cells by the use of the calibration curve mentioned above. Subsequently, the decrease in algal cells over two hours was estimated by calculating the slope of the regression line of the ln-transformed cell numbers as a function of time (in min; after Stier et al., 2015). Finally, to assess the clearance rate in mL min^{-1} we multiplied the slope of each individual regression by -1000, to account for the 1000 mL volume of water in which the mussels were kept during the measurements. Some mussels did not filter at all during the measurements and therefore a separate category (successful filtration: yes/no) was used as a random effect in the clearance rate mixed model to take this variation into account (see section *Statistical analysis*).

Body condition and growth

At the termination of the experiment, we dissected all mussels to check for the presence of *M. orientalis* in "infected" mussels and absence in the "control" mussels. Mussel tissue was compressed between glass slides and examined under a stereo microscope (magnification 10-80x) to account for all parasitic copepods, including larvae and juveniles (Gee and Davey, 1986a).

We separated the shells from the mussel tissue and extracted adult copepods from the intestines. Larvae were left in the tissue, as these were too small to handle without disturbing the mussel flesh. We froze (-20 °C for at least 24 hours) and freeze-dried the mussel tissue (48 hours) to determine dry weight. Condition index was determined as CI = DW L^{-3}, where DW is the dry weight (mg) of the tissue and L is the final shell length (cm, after Petersen et al., 2004). At termination of the experiment, the length of each individual mussel shell was measured to the nearest mm with callipers. Growth was then calculated by extracting initial length from the final length of each mussel.

Statistical analysis

All statistical analyses were performed using the statistical software package R (R Development Core Team, 2015) and model assumptions were confirmed using diagnostic model plots (Zuur et al., 2010). P values of < 0.05 were considered as significant.

Natural infections in the field

The condition index was log transformed to improve normality of the data. Subsequently, the difference in body condition in infected (n = 18) and uninfected mussels (n = 21) was analysed with a Student's t-test.

Failed controlled infections

Experimental blocks found to contain mussels with failed infections (exposed to larvae but not found to be infected at the end of the experiment), or unanticipated infections (found to be infected despite not having been exposed to larvae) were excluded from the analysis to preserve a balanced design with a complete dataset. In the first batch of the experiment, four mussels (out of 34 mussels exposed to larvae) remained uninfected and one mussel was unintentionally infected, while in the second batch infection success was lower and seven (out of 30) mussels where left uninfected. After removing all blocks with failed infections, 12 blocks were left for the first and 10 blocks for the second batch of the experiment.

Laboratory experiment

Each of the two experimental batches was analysed separately, but similar models were used for both batches. To test for effects of the parasite on clearance rate of the mussels, we applied a square root transformation on clearance rate data and used a linear mixed model (lmm; lmer function from the package lme4; Bates et al., 2015) including infection status (infected/uninfected), food level (high/low) and time plus their interactions, and experimental block as fixed factors. Individual mussels and successful filtration (see section *Measurement of clearance rate, body condition and growth*) were included as random effects. We used a similar model to investigate the effect of infection intensity (number of *M. orientalis* individuals per infected mussel) on clearance rate of infected mussels, but instead of infection status (infected/uninfected) we used the number of *M. orientalis* individuals as predictor in the model. For all these mixed models, p-values were obtained by comparing the full model (with all fixed effects) against a reduced model (without the fixed effect in question) with a likelihood ratio test.

Condition index was log transformed to improve normality of the data. To test for effects of the parasite on the condition of blue mussels, we applied a general linear model (ANOVA) with infection status (infected/uninfected), food level (high/low), their interaction and the blocking factor as explanatory variables. Again, we used a similar model to investigate the effect of infection intensity on the condition index of infected mussels, but we replaced infection status (infected/uninfected) with *M. orientalis* intensity.

Finally, to test for the effects of the parasite on mussel growth, we first modelled growth against mussel length at the start of the experiment with a linear model. We took the residuals from this model as a proxy for growth (corrected for initial length) and subsequently used another general linear model to test for the effects of infection status (infected/uninfected), food level (high/low), their interaction, and experimental blocking as explanatory variables. Finally, we tested the effect of intensity of infection on (corrected) growth of infected mussels, by using a similar linear model where we replaced the infection category by numbers of *M. orientalis*.

Results

Natural infections

Mytilicola orientalis prevalence in blue mussels (*Mytilus edulis*, 30-35 mm; the size category used in the experiments) on a mixed mussel (*M. edulis*) and Pacific oyster (*Crassostrea gigas*) bed on a tidal flat on the east coast of Texel (Vlakte van Kerken) was 50% with a mean (± SD) intensity of 3.0 (± 1.6) individuals per infected host. Naturally infected mussels tended to have 20% lower condition indices than uninfected mussels, however the difference was only marginally significant (t = 8.880, p = 0.068; Fig. 7.1).

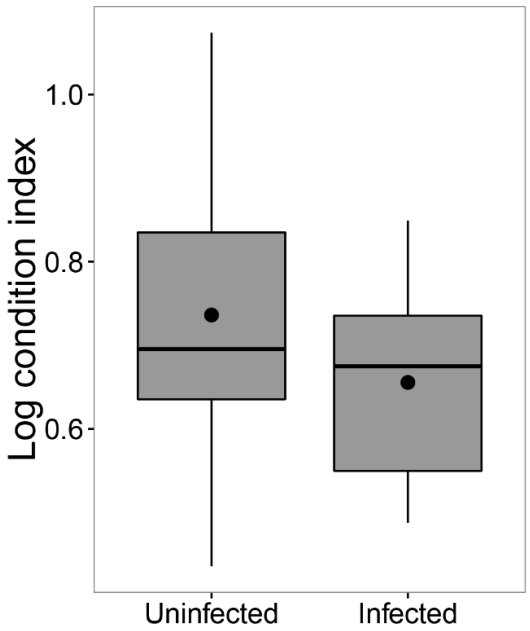

Fig. 7.1 Condition index (log transformed) of uninfected mussels (n = 21) and mussels infected with *Mytilicola orientalis* (n = 18) collected on a mixed blue mussel (*Mytilus edulis*) and Pacific oyster (*Crassostrea gigas*) bed on a tidal flat on the east coast of Texel (Vlakte van Kerken, Texel, The Netherlands). The black dots represent the mean condition indices of each group. Note the truncated y-axis.

Success of artificial infections

Hatching success

Dissection of 140 mussels from a mixed mussel and oyster bed on the east coast of Texel (Vlakte van Kerken) produced 60 egg sacs (prevalence of gravid females 43%). Time from egg sac extraction to hatching of copepod larvae was highly variable and ranged from immediate hatching to eight days after extraction, with an average of 4.4 days. At early phases the eggs were opaque (Fig. 7.2 A), but when close to hatching, the eggs became transparent and the red eye spots of the larvae became visible through the egg case (Fig. 7.2 B). All eggs within an individual egg sac developed at similar rates, however hatching success ranged from 0 - 100% and 26.1% of the eggs failed to hatch at all. The nauplius phase (Fig. 7.2 C) lasted a day maximum and infective copepodite I larvae (Fig. 7.2 D) appeared on average 4.8 days after egg extraction, though the earliest larvae metamorphosed within two days. After eight days, larval survival declined and the collection period was terminated. The nauplii were 200 - 220 µm in length and the copepodite I (infestive) stages were 240-290 µm long. On average 50 copepodite larvae successfully emerged from a single female's egg sac pair (i.e. approximately 25 per egg sac), although the maximum recorded was over 200 copepodites in a pair.

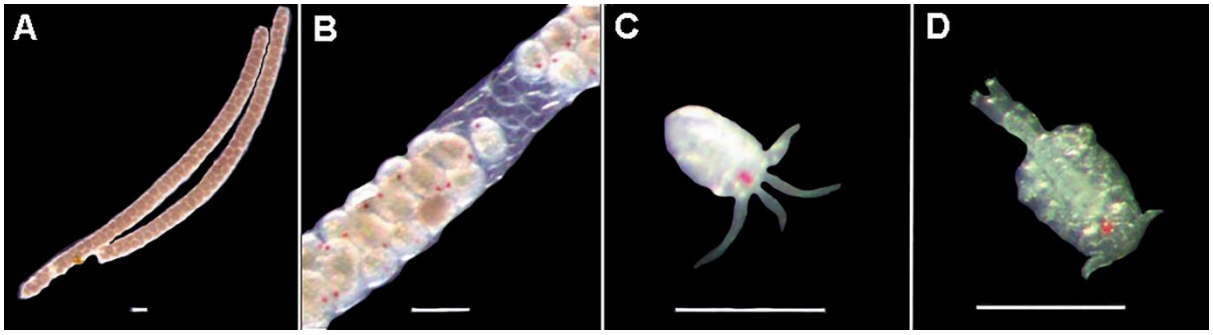

Fig. 7.2 Developmental phases of *Mytilicola orientalis*: **A)** a pair of egg sacs, **B)** eggs about to hatch (note the red eye spots), **C)** nauplius and **D)** infective copepodite I larva. The white scale bars denote 200 µm.

Infection success

Infection success differed among the two experimental batches (Table 7.1). The first batch of mussels had a higher infection success (88%) than the second batch (76%; Table 7.1). The maximum number of individual *M. orientalis* found in a single mussel was 12. Average intensities of artificial infections in both batches were comparable to those in the field (mean ± SD; Batch 1: 3.0 ± 2.4, Batch 2: 3.5 ± 3.2). Like adults, juvenile copepods were found in the digestive tract in proximity to the stomach of blue mussels and were approximately max. 2 mm long at termination of the experiment (8-9 weeks; Fig. 7.3). Therefore, the copepodites had almost grown ten times as large, at a rate of about 30 µm day^{-1} since they infected their host. As the copepods were not yet grown to full size of maturity, it was impossible to determine the sex of the parasites. The uninfected control mussels were confirmed to be free of infection, except for one mussel in the first batch that was infected with two adult female *M. orientalis*.

Table 7.1 Infection protocol and infection results for the two batches in the experiment, with the batch number, addition of food during parasite exposure, the unit and duration in which exposure took place, the experimental unit and duration, infection success (number of successfully infected mussels out of the total number of exposed mussels) and mean intensity (± SD) of successfully infected mussels.

Batch	Food addition during exposure	Exposure unit and duration	Experimental unit and duration	Infection success	Mean intensity (± SD)
1	Yes	100 mL 24 h	1000 mL 8 weeks	88% (30/34)	3.0 ± 2.4
2	No	1000 mL 9 weeks (entire experiment)		76% (23/30)	3.5 ± 3.2

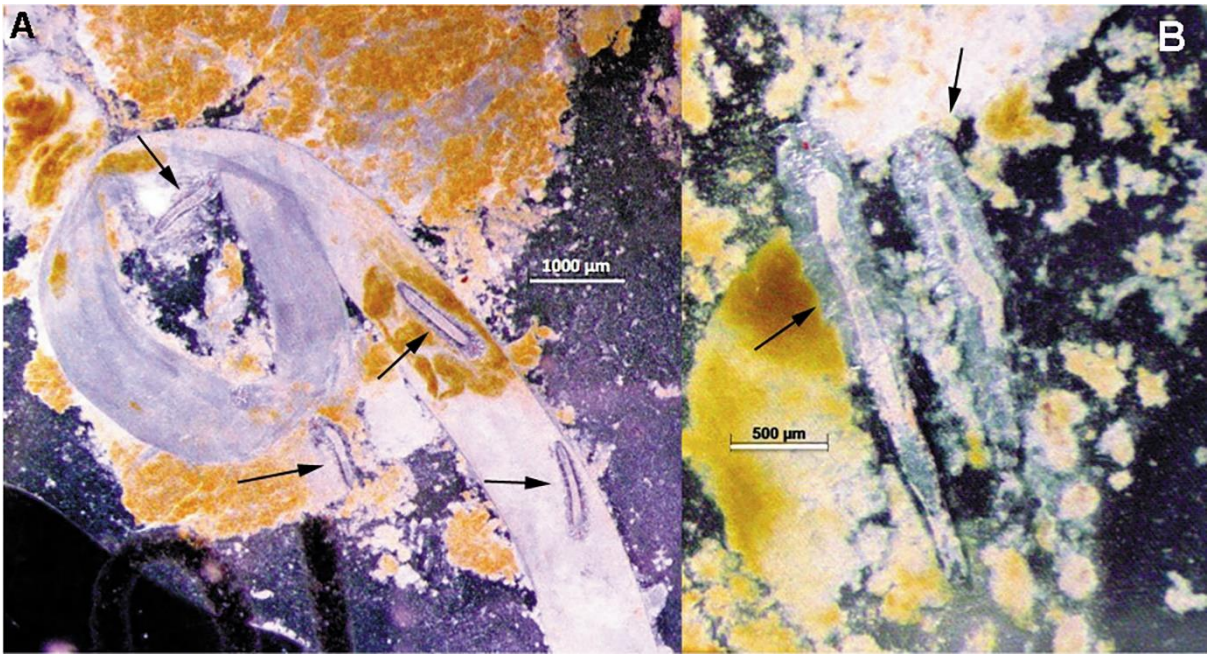

Fig. 7.3 Developmental status of *Mytilicola orientalis* infections after approximately **A)** 5.5 weeks (scale bar denotes 1000 µm) and **B)** 8 weeks (scale bar denotes 500 µm) after exposure to larvae.

Effects on clearance rate

In both experimental batches, no mortality occurred in infected and uninfected mussels. For either batch in this experiment we did not find significant overall effects of infection status or food limitation on mussel clearance rate (Table 7.2; Fig. 7.4). However, clearance rate significantly differed over time in the second (lmm; $p < 0.001$; Fig. 7.4 B; Table 7.2) but not in the first batch ($p = 0.722$; Fig. 7.4 A; Table 7.2) of the experiment. This difference probably results from the relative high clearance rates in the first week and relative lower clearance rates in week 7 of batch 2 in comparison to batch 1 of the experiment (Fig. 7.4). When testing for the effects of infection intensity upon infected mussels, we found no significant results for any of the factors in the first batch, but a marginally significant interaction between food level and infection intensity ($p = 0.066$; mussels with higher infection intensities fed under low food conditions tended to have lower clearance rates) and a significant effect of time ($p < 0.001$; Table 7.2) in the second batch of the experiment.

Table 7.2 Results of linear mixed models that tested for effects of infection with *Mytilicola orientalis* and food level on clearance rate (square root transformed) of blue mussels in the two batches of the experiment. The infection model included the effects on all mussels, while the intensity model only tested the effect on infected mussels. P-values were obtained by comparing the full model (with all fixed effects) against a reduced model (without the fixed effect in question) with a likelihood ratio test.

Data selection	**Effect**	**Factor**	**Batch 1**					**Batch 2**				
			β	*SE*	*t*	χ^2	*P*	*β*	*SE*	*t*	χ^2	*P*
All mussels	Fixed	Intercept	-1.634	1.042	1.570			2.321	1.037	2.238		
		Food	-0.370	0.311	-1.187	2.067	0.724	0.066	0.293	0.226	2.134	0.710
		Infection	-0.073	0.312	-0.233	0.566	0.967	0.024	0.293	0.081	2.502	0.664
		Time	-0.042	0.041	-1.028	2.075	0.722	-0.164	0.035	-4.637	70.846	< 0.001
		Block	0.004	0.008	0.447	0.245	0.621	-0.005	0.015	-0.316	0.100	0.752
		Food * Infection	0.187	0.441	0.424	0.467	0.792	-0.081	0.414	-0.195	1.938	0.380
		Food * Time	0.074	0.057	1.287	1.852	0.396	0.024	0.050	0.483	0.329	0.849
		Infection * Time	0.016	0.057	0.279	0.391	0.822	0.008	0.050	0.163	0.421	0.810
		Food * Time * Infection	-0.048	0.081	-0.594	0.352	0.553	-0.040	0.071	-0.560	0.313	0.576
	Random	Variables	Var.	SD				Var.	SD			
		Ind. mussels	0.000	0.000				0.034	0.184			
		Filtration success	2.053	1.433				1.728	1.314			
		Residual	0.540	0.735				0.747	0.864			
Infected mussels only	Fixed	Intercept	1.854	1.071	1.730			1.297	1.260	1.030		
		Intensity	-0.076	0.069	-1.093	2.834	0.586	0.063	0.120	0.525	6.254	0.181
		Food level	-0.765	0.554	-1.381	4.040	0.401	0.150	0.457	0.328	6.290	0.179
		Time	-0.098	0.061	-1.594	3.681	0.451	-0.208	0.057	-3.666	36.219	< 0.001
		Block	0.010	0.013	0.760	0.702	0.402	0.026	0.025	1.053	1.077	0.299
		Food * Time	0.181	0.101	1.795	3.383	0.184	0.087	0.075	1.155	1.496	0.473
		Intensity * Time	0.015	0.013	1.212	2.600	0.458	0.020	0.020	1.007	5.445	0.142
		Food * Intensity	0.116	0.146	0.793	2.117	0.347	-0.043	0.136	-0.320	5.444	0.066
		Food * Time * Intensity	-0.033	0.027	-1.226	4.288	0.368	-0.025	0.022	-1.138	5.656	0.226
	Random	Variables	Var.	SD				Var.	SD			
		Ind. mussels	0.002	0.050				0.051	0.227			
		Filtration success	2.041	1.429				1.947	1.395			
		Residual	0.518	0.720				0.703	0.839			

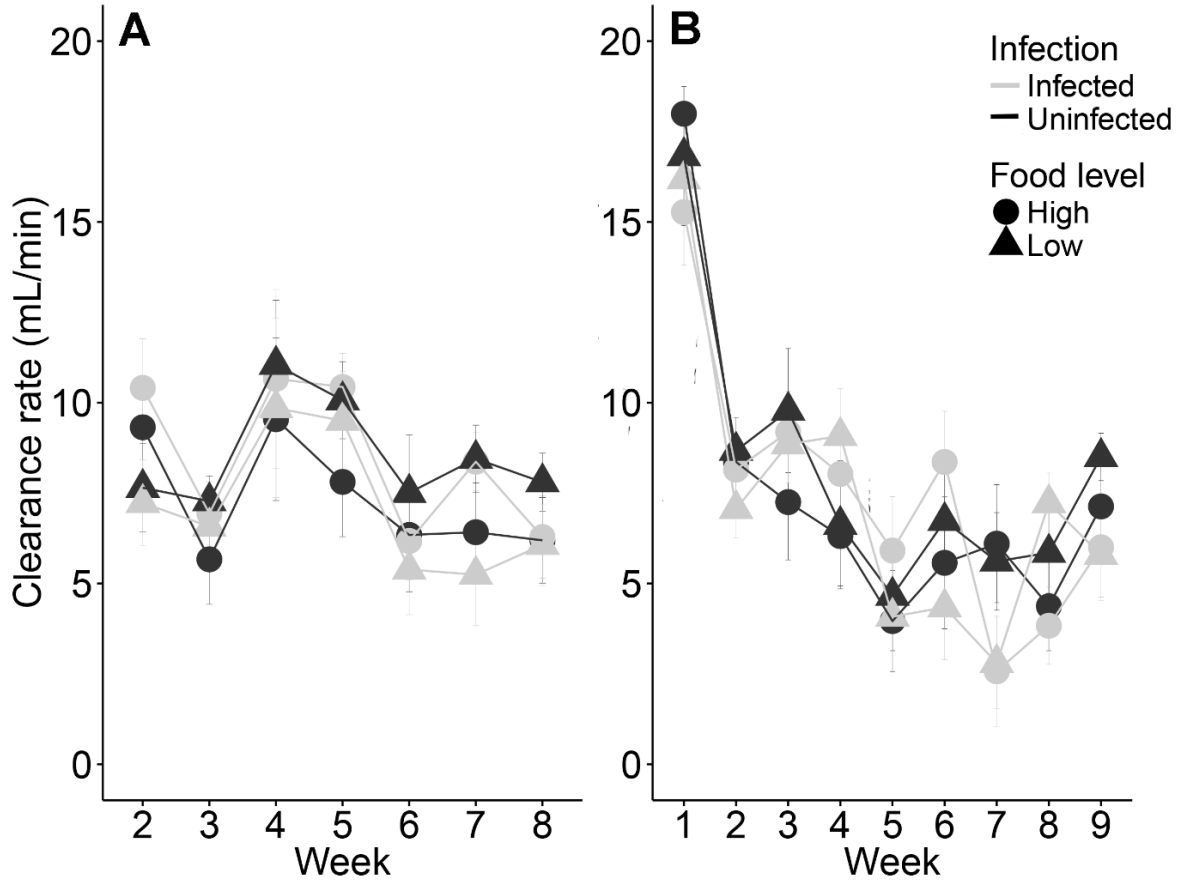

Fig. 7.4 Mean clearance rate (± SE) of uninfected blue mussels (grey) and mussels infected with early stages of *Mytilicola orientalis* (< 9 weeks; black) fed under high (circles) and low (triangles) food conditions. Clearance rates were measured weekly after exposure to infestive larvae for each of the four treatment groups in **A)** the first and **B)** second batch of the experiment.

Effects on body condition

Infected mussels had significantly lower condition indices (Batch 1: 11%, Batch 2: 13%) than uninfected mussels (LRT; Batch 1: $p < 0.05$, Batch 2: $p < 0.05$, Fig. 7.5, Table 7.3). Furthermore, infected mussels kept under low food levels had the tendency to have lower condition indices, while uninfected mussels had slightly increased condition indices (Fig. 7.5). However, in both batches, the effect of food level as well as the interaction between infection and food level was not significant (Table 7.3).

In additional analyses, where we tested for an effect of *M. orientalis* intensity and food limitation on the condition index of infected mussels, we found different results for both batches. In the first batch, mussels with higher infection intensities had slightly higher condition indices than mussels with lower infection intensities ($p < 0.05$), but this result was not significant in the second experimental batch (Table 7.3). Additionally, the block factor was significant in the first batch ($p < 0.01$), but again not significant in the second batch of the experiment. Finally, we did not find any significant effects of food level or the interaction between intensity and food level in both batches of the experiment (Table 7.3).

Table 7.3 Results of linear models that tested for effects of infection with *Mytilicola orientalis* and food level on blue mussel condition (log transformed) in the two batches of the experiment. The effects of infection and food level were tested for all mussels, while the effect of parasite intensity was only tested for infected mussels in the experiment.

Data selection	Factor	Batch 1					Batch 2				
		df	*SS*	*MS*	*F*	*p*	*df*	*MS*	*SS*	*F*	*p*
All mussels	Food	1	0.003	0.003	0.317	0.577	1	0.001	0.001	0.074	0.789
	Infection	1	0.045	0.045	4.153	< 0.05	1	0.039	0.039	4.956	< 0.05
	Block	11	0.142	0.013	1.183	0.336	9	0.148	0.016	2.101	0.066
	Infection * Food	1	0.006	0.006	0.515	0.478	1	0.003	0.003	0.437	0.514
	Residuals	33	0.361	0.011			27	0.211	0.008		
Infected mussels only	Intensity	1	0.032	0.032	5.374	< 0.05	1	0.001	0.001	0.078	0.788
	Food	1	0.005	0.005	0.833	0.385	1	0.001	0.001	0.079	0.786
	Block	11	0.312	0.028	4.774	< 0.05	9	0.102	0.011	0.890	0.575
	Intensity * Food	1	0.003	0.003	0.465	0.512	1	0.002	0.002	0.144	0.715
	Residuals	9	0.054	0.006			7	0.090	0.013		

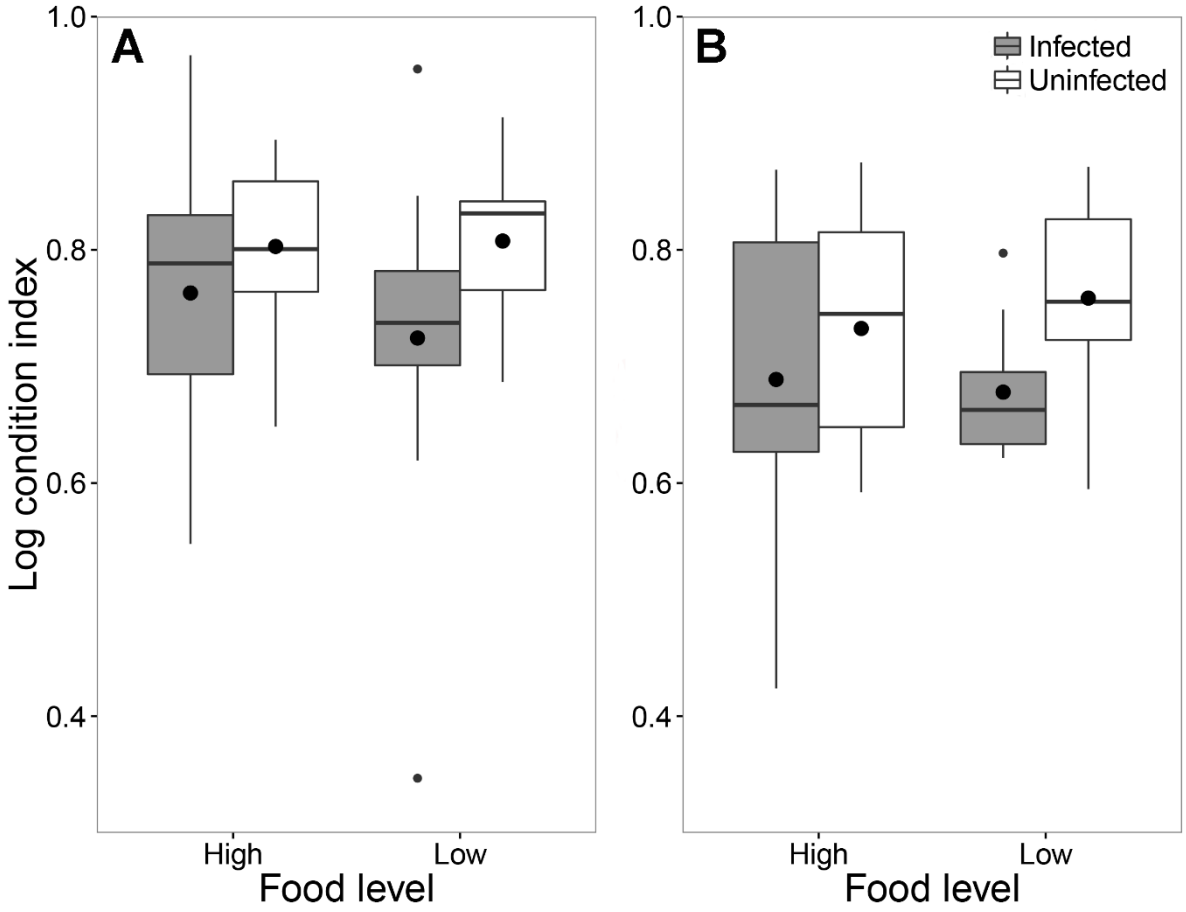

Fig. 7.5 Boxplots of the condition index (log transformed) of mussels in **A)** the first and **B)** second batch of the experiment that were either infected (grey) or uninfected (white) with early stages of *Mytilicola orientalis* (< 9 weeks) and kept under high or low food level conditions during the nine weeks of the experiment. The black dot within each box indicates the group's mean condition index. Note the truncated y-axis.

Effects on growth

Mean growth (± SE) was 0.56 (± 0.05) mm in the first batch and 0.49 (± 0.07) mm in the second batch of the experiment, which is an average of about 0.01 mm day^{-1}. In both batches, growth (corrected for initial shell length) of mussels was not significantly affected by *M. orientalis* infection, food level, the interaction between those variables and the blocking factor (Table 7.4). Furthermore, among only the infected individuals in both batches, we did not detect any significant effect of infection intensity, food level, an interaction between those terms or an effect of experimental blocking on the growth of infected mussels in either batch (Table 7.4).

Table 7.4 Results of linear models that tested for effects of infection with *Mytilicola orientalis* and food level on blue mussel growth (corrected for initial length) in the two batches of the experiment. The effects of infection and food level were tested with linear models for all mussels, while the effect of parasite intensity tested was only tested for infected mussels in the experiment.

Data	Factor	Batch 1					Batch 2				
		df	*SS*	*MS*	*F*	*P*	*df*	*MS*	*SS*	*F*	*P*
All mussels	Food	1	0.178	0.178	1.274	0.267	1	0.137	0.137	0.869	0.360
	Infection	1	0.095	0.095	0.681	0.415	1	0.053	0.053	0.337	0.567
	Block	11	1.029	0.094	0.670	0.756	9	2.519	0.280	1.770	0.121
	Infection * Food	1	0.263	0.263	1.881	0.180	1	0.440	0.440	2.781	0.107
	Residuals	33	4.607	0.140			27	0.158	0.158		
Infected mussels only	Intensity	1	0.054	0.054	0.366	0.550	1	0.082	0.082	0.305	0.598
	Food	1	0.193	0.193	1.304	0.262	1	0.076	0.076	0.281	0.612
	Block	11	0.997	0.091	0.612	0.805	9	1.615	0.180	0.665	0.720
	Intensity * Food	1	0.042	0.042	0.285	0.597	1	0.009	0.009	0.034	0.858
	Residuals	33	4.886	0.148			7	1.890	0.270		

Discussion

This study is the first to experimentally test for the effects of the invasive parasitic copepod *Mytilicola orientalis* (which has recently spilled over from invasive Pacific oysters *Crassostrea gigas*) on native blue mussels (*Mytilus edulis*). By using controlled infections and laboratory experiments, we found significant negative effects of infection with (juvenile stages of) the invasive parasite on the condition of mussels. Naturally infected mussels from the field also had lower condition than uninfected mussels, but the difference was only marginally significant. Evidence for parasite effects on mussel clearance rates was limited, and no effects on mussel survival and growth were found.

The detrimental effect of especially early stages of *Mytilicola* infections has been previously suggested (for the congeneric *M. intestinalis*; Korringa, 1950; Dethlefsen, 1985) due their presence in the ramifications of the digestive gland (Campbell, 1970). This is the digestive organ in molluscs and infections may compromise its functioning. Additionally, the growth rate of especially young individuals of any species (in our experiment copepods grew on almost 10 times as large over 8 weeks (32 µm day^{-1}), is usually larger than that of adults, requiring enormous amounts of energy. As stable isotope analyses suggest that *Mytilicola* feeds on host tissue (Gresty and Quarmby, 1991), the high energy demand of the growing copepods can be expected to lead to a significant loss of tissue, ultimately resulting in lower host condition (11-13% in our experiments). Moreover, this feeding activity will incur other energetic costs: when *Mytilicola* feeds on host tissue, the resulting metaplasia of the host gut epithelium (Moore et al., 1977) needs to be repaired, which is an energetically demanding process for the host and likely

to reduce host condition. When *Mytilicola* matures, the effects of the parasite may become less severe as the copepods move away from the digestive gland and migrate further down the digestive tract (Grainger, 1951; Gee and Davey, 1986a). A decrease in harmfulness with parasite age may also explain that we only found a marginally significant difference in condition between infected and uninfected mussels in the field, as those infections consisted of a mix of young and older stages of *M. orientalis*. Generally, adverse effects of *M. orientalis* on host condition have also been reported for oysters (e.g. *Ostrea lurida*: Odlaug, 1946; *Crassostrea gigas*: Katkansky et al., 1967) and are known to increase with parasite intensity (Katkansky et al., 1967). However, in our experiments we could not find a general trend of declining mussel condition with infection intensity, as the two experimental batches gave contrary results in this respect. The presence of sometimes high intensities of remaining larvae in the digestive tissue (too miniature to be taken out without removing tissue) could have positively influenced the weight measurements and thereby contributed to the observed slight positive effect of infection intensity on mussel condition in the first batch of the experiment. Nevertheless, the general negative effect of infections with early stages of *M. orientalis* on mussel condition suggests that native mussels are experiencing negative effects due to the spillover of this invasive parasite. As condition is related to reproductive output in mussels (e.g. Bayne et al., 1983) and because prevalence of *M. orientalis* in mussels is often high (up to 63%; Goedknegt et al., 2017), the impact of this invader may extend beyond individual infected mussels, however, this remains to be investigated.

In contrast to the adverse effects of juvenile *M. orientalis* on mussel condition, we only found limited evidence of negative effects of *M. orientalis* on clearance rates of mussels. This is also in contrast with a previous study that observed a reduced filtration capacity in mussels infected with trematode metacercariae, which encyst in mussel gills and palps and interfere with filtration (Stier et al., 2015). However, as *Mytilicola* resides in the mussels' intestines it may not directly impact gill function in the same way as trematode metacercariae. Instead, *Mytilicola* infections may only indirectly affect filtration by influencing host energy requirements and expenditure. However, we did not find evidence for such effects in our experiment and therefore negative effects of *Mytilicola* infections on mussel clearance rates seem to be limited. We acknowledge that our inference in this respect might have been hampered due to the relatively large variation in clearance rates we observed within and among mussels over time, especially in the second batch of the experiment. Part of this variation is explained by the mussels that did not filter during our experiment, which we included as a random effect in the model. Overall, observed clearance rates were relatively low and in many cases dropped to less than 10 mL/min, which is lower than filtration rates previously reported for mussels under comparable algal concentrations (Clausen and Riisgård, 1996; Stier et al., 2015). The underlying reasons for these low values are not known but suggest that that mussels were not optimally adjusted to our experimental conditions. The low clearance rates may also explain the limited growth of all mussels (on average 0.01 mm day^{-1}) and the subsequent lack of negative effects of *M. orientalis* on mussel growth. Nevertheless, a lack of an adverse effect of *M. orientalis* on growth is in correspondence with observational studies of Pacific oysters (Katkansky et al., 1967; Steele and Mulcahy, 2001). Finally, a negative effect of the parasite on mussel survival could have been expected given that it's congeneric *M. intestinalis* has been considered to be the causative agent of mussel mass mortalities in the past (Korringa, 1968; Blateau et al., 1992). However, in our experiment there was no mortality of mussels among experimental treatments, illustrating the sub-lethality of the parasite that has also previously been shown for Pacific oysters (Katkansky et al., 1967).

In contrast to our expectation, we did not find clear evidence that food shortage exacerbated the effects of *Mytilicola* infections. Only in the second batch of the experiment we observed a marginally significant interaction between infection intensity and food level, where mussels infected with more parasites but fed under low food conditions had slightly lower clearance rates. This indicates that potential synergistic exacerbating effects are weak, and that only mussels with high infection intensities living under low food conditions might suffer from lower clearance rates. However, this result was not found in the first batch of the experiment and it had no consequence for differences in mussel condition between food levels. Conceivably, the chosen food regimes did not differ enough to be able to detect stronger exacerbating effects. The limited growth of mussels in our experiment, even in the high food-no infection treatment, also suggests this possibility. Therefore, future studies may investigate the harmfulness of the parasite under more extreme food conditions.

In general, controlled infections of hosts with *Mytilicola* infestive larval stages proved to be an effective method, which can be applied in subsequent studies as they will help to overcome the lack of strong inference in earlier correlative studies on both *Mytilicola* species. Here, we have developed a successful technique to harvest the invasive parasite *M. orientalis* and to infect its new blue mussel host under laboratory conditions. The lack of mortality of mussels among treatments during the entire experiment, implies, besides suggesting only sublethal effects of the parasite, that our experimental procedures were non-lethal. Furthermore, we have documented, for the first time, the maturation of *M. orientalis* larval stages from the moment of hatching to the development of the infective stage, which typically took less than one week under the relatively high temperatures used to increase development speed (i.e. the planktonic phase is likely to be longer in natural populations). Our infection methods were successful (success rate 71-88%) and we achieved mean intensities (approx. 3 copepods per infected host) that were similar to intensities observed in natural populations of infected mussels. As the results of our varying infection techniques only marginally differed between batches, the addition of food during parasite exposure and the size of the infection containers do not appear to drastically affect the outcome of laboratory infections. Given that Gee and Davey (1986a) estimated a maturation period of 70.8 (± 16.6, 95% confidence interval) days at 14-18°C and just 8.3 (± 4.1) days at 18-22 °C for *M. intestinalis*, we expected our experiments to provide ample time for the parasites to achieve maturity. However, our screenings unexpectedly revealed almost exclusively juvenile *M. orientalis* after nine weeks in the experiment, carried out at 18 °C. This may indicate that *M. orientalis* maturation times are significantly longer than *M. intestinalis* due to a lower tolerance for cool temperatures and further studies will be needed to determine developmental times of the parasite at various temperatures.

In conclusion, this is the first study in which controlled laboratory infections with the invasive copepod *M. orientalis* were performed on its new native blue mussel host. We discovered that infections with early stages of the copepod (up to nine weeks) lead to lower condition of infected mussels. As our study was performed with juvenile stages of the parasitic copepod, potential impacts of adult parasites remain to be investigated. In addition, in our experimental study, we challenged the mussels only with two stressors (infection with *M. orientalis* and limiting food conditions). However, for mussels living on natural mussel beds, stressors may be more diverse and severe (e.g. extreme temperatures, infections with multiple parasite species, resource competition with other species), opening perspectives for future studies. Such studies will be important to identify the full range of indirect effects of invasive oysters and other invasive species on native biota via parasite co-introductions and subsequent parasite spillover.

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