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Impact of the invasive parasitic copepod *Mytilicola orientalis* on native blue mussels *Mytilus edulis* in the western European Wadden Sea

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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 (*Magallana gigas*) co-introduced the parasitic copepod *Mytilicola orientalis* that spills over to native blue mussels (*Mytilus edulis*) and other native bivalves. In this study, we investigated the impact of *M. orientalis* infections on blue mussels by conducting laboratory experiments 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 a significantly lower body condition (11-13%) compared to uninfected mussels after nine weeks of infection. However, naturally infected mussels from a mixed mussel and oyster bed did not significantly differ in body condition compared to uninfected mussels. Contrary to effects on mussel condition, we did not find an effect of experimental infections on clearance rates, shell 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. The results of this study call for the integration of such parasite-mediated indirect effects of invasions in impact assessments of invasive species.

Keywords

controlled infection, *Magallana gigas*, invasive species, Pacific oyster, parasite co-introduction, parasite spillover
**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, one of being the co-introduction of a parasite with an invasive host species (parasite co-introduction; Lymbery et al. 2014). Consequently, in the invaded range, a 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). Such parasite spillover events also occur in marine ecosystems and can lead to emerging diseases and subsequent mass mortalities of native hosts with, in several cases, knock-on effects on native communities and ecosystems (Goedknegt et al. 2016).

Parasite co-introduction and spillover have also occurred in the course of the introduction of the Pacific oyster, *Magallana* (previously *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 digestive tract 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 wild native blue mussels (*Mytilus edulis* Linnaeus, 1758), common cockles (*Cerastoderma edule* Linnaeus, 1758), Baltic tellins (*Limecola* (formerly *Macoma*) *balthica* Linnaeus, 1758) and European flat oysters (*Ostrea edulis* Linnaeus, 1758), indicating several spillover events (His 1977; Stock 1993; Pogoda et al.
In particular, native blue mussels 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. 2017a). Native blue mussels are also infected by *Mytilicola intestinalis* Steuer, 1902, a related parasite species described from the Mediterranean Sea (Steuer 1902), which was first found in the Wadden Sea in 1938 (Caspers 1939) and that has a similar direct life cycle as *M. orientalis* (Caspers 1939; Grainger 1951; Dethlefsen 1985; Gee & Davey 1986). *Mytilicola intestinalis* became infamous as the ‘red worm disease’, because it was thought 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 to support this hypothesis is rare. Historically, especially juvenile stages of *M. intestinalis* were held responsible for mortalities of mussels (Korringa 1950; Dethlefsen 1985) because of 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 their egg yolk, the only food source available during the pelagic larval phase (Grainger 1951). This increases the chance for mussels to be negatively affected by juvenile parasites. For mature *M. intestinalis*, lethal and sublethal effects on important host fitness components such as body condition, filtration rate and reproduction do not seem to be one-directional and have been controversially discussed (Lauckner 1983). Only recently, controlled infection experiments have been conducted with *M. intestinalis* and blue mussels, in which a reduction in blue mussel dry weight was found as a result of infection by the parasite in sympatric parasite-host populations (Feis et al. 2016). Similarly, although *M. orientalis* is generally considered a serious pest (Holmes and Minchin 1995) and is registered on the list of 100 worst invaders of the Mediterranean (Streftaris and Zenetos 2006), studies on its lethal and sublethal effects on *M.
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4 gigas (Katkansky et al. 1967; Deslous-Paoli 1981; De Grave et al. 1995; Steele & Mulcahy 2001) and Ostrea lurida Carpenter, 1864 (Odlaug 1946) have been inconclusive and they have generally lacked experimental approaches. The effects of M. orientalis on its new host, the blue mussel, have not been studied to date. A potential effect of M. orientalis on native blue mussels is likely to be modified by environmental factors and species interactions (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 the 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 effect of Mytilicola spp. infections on hosts at low food levels is lacking.

In this study, we investigated the effects of M. orientalis infection on the native blue mussel M. edulis by conducting a laboratory experiment that used controlled infections of mussels with larval stages of the copepods. Following the hypothesis of Moore et al. (1977), we also tested whether the potential adverse effects of infection on survival, clearance rate, body condition and shell growth of blue mussels intensified under low food conditions. In addition, we collected mussels in the field to determine natural infection levels and to investigate the effect of Mytilicola infections on mussel body condition in a natural environment. This combination of lab and field investigations allowed us to assess the impact of the spillover of the invasive parasite M. orientalis from invasive Pacific oysters on native blue mussels.

Material and Methods
**Field survey**

To determine natural prevalence (the proportion of infected individuals) and mean infection intensity (mean number of parasites in infected mussels) of *Mytilicola orientalis* in blue mussels in the wild, we collected 30 mussels of 30-50 mm length from a mixed bed of Pacific oysters and blue mussels located on the Vlakte van Kerken, a tidal flat on the east coast of the island of Texel (Figure 1) in the western European Wadden Sea (southern North Sea) on 11 June 2014. Additionally, we collected from this bed another 39 mussels (34-54 mm) to analyse the effect of infection status (infected/uninfected) on mussel body condition.

**Experimental infections**

Uninfected mussels (30-35 mm; *n* = 150) for the experiments were haphazardly collected from basalt groins on the north-west shore of the Dutch mainland (Julianadorp, Figure 1) on 11 September 2014. Previous explorations by M. A. Goedknegt had shown that *Mytilicola* spp. does not occur at this location, which was verified by haphazardly sampling and screening 30 additional mussels, which were all found to be free of infection. Any epifauna (mostly barnacles) on the mussels was carefully removed from the shells to ensure that copepod larvae could infect mussels without being eaten or physically obstructed during experimental infections (Johnson & 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* Parke, 1949 culture, or alternatively with Phyto-Feast® when fresh culture was unavailable (on average once a week).
To acquire a source for *M. orientalis* larvae, mussels (*n* = 140) were haphazardly collected from a mixed bed with known *Mytilicola* infections located on a tidal flat on the east coast of the island of Texel (Figure 1) on 22 August 2014. Within two days of collection, mussels were dissected and gravid *M. orientalis* 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 & Davey (1986). When larvae had developed into the infective copepod I stage (Gee & Davey 1986), uninfected mussels (*n* = 74) were exposed to the larvae.

Before exposure to infective larvae, the shell length (maximum anteroposterior axis) of each mussel was measured with callipers to the nearest mm. Because of variation in larval hatching and development time, insufficient larvae were available to infect all mussels at once, ultimately resulting in two temporal experiments of infected mussels. Individual mussels in the first experiment (*n* = 34 mussels) were exposed to parasites in a 100-mL cup and after 24 h, mussels and filtered sea water were transferred to a 1000-mL container for the following eight weeks of the experiment. For individual mussels in the second experiment (*n* = 30), 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 infective 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 filtered sea water. To promote filtration and uptake of
infective larvae, small amounts of algal culture (*I. galbana*) were added to the sea water during
exposure of mussels of the second experiment. Five extra mussels for each of the two
experiments were artificially infected and sacrificed for examination of larval development at
mid-way points during the experiments. Control (uninfected) mussels (Experiment 1: \( n = 34 \),
Experiment 2: \( n = 30 \)) were treated identically to exposed mussels within each experiment
(transfer of filtered sea water and small amounts of algal culture) but without the addition of
copepodites.

*Experimental set-up*

The experiment was set up immediately after exposing the mussels to the parasite larvae, which
was for the first experiment on 10 September 2014 and the second experiment on 7 October
2014. 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 each containing one individual mussel. Thus, each block contained a replicate of
the following treatments: uninfected mussel – low food level, infected mussel – low food level,
uninfected mussel – high food level, and infected mussel – high food level. The first experiment
contained 17 of these replicated blocks (\( n = 68 \) mussels in total), while the second experiment
contained 15 replicated blocks (\( n = 60 \) mussels in total). 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 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, on
average once per week, 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. Only fresh algal culture was provided on days when clearance rates were measured.

After dissections at the end of the experiment, we found that some experimental blocks contained 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). In these cases, affected blocks were excluded from the analysis to preserve a balanced design with a complete dataset. In the first experiment, four mussels (out of 34 mussels exposed to larvae) remained uninfected and one mussel was unintentionally infected, while in the second experiment infection success was lower and seven (out of 30) mussels were uninfected. After removing all blocks with failed and unanticipated infections, 12 blocks were left for the first experiment ($n = 48$ mussels) and 10 blocks for the second experiment ($n = 40$ mussels).

**Measurement of clearance rate, body condition and shell growth**

**Clearance rate**

We conducted the first clearance rate measurements of the mussels of the first experiment one week after exposure to *M. orientalis* larvae, while the clearance rate of mussels of the second experiment 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.

Clearance rate was assessed by means of the indirect clearance method (Riisgård 2001; Petersen et al. 2004). One day prior to the measurement, all containers were refreshed with filtered seawater and mussels were checked for survival (severely gaping mussels in smelly water were assigned as dead). 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 Relative Fluorescence Units (RFU) using a Trilogy® Laboratory Fluorometer (Turner Designs), which 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 created a calibration curve to calculate the amount of algal culture needed to create an initial starting concentration of $13 \times 10^3 \text{–} 14 \times 10^3$ 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 by 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. 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.
Body condition and shell growth

The experiments ran for eight (Experiment 1) and nine weeks (Experiment 2) and immediately after termination of the experiments all mussels were measured, screened for presence of *M. orientalis* larvae, frozen, freeze-dried and weighed to assess body condition. 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. Mussel tissue was compressed between glass slides and examined under a stereo microscope (magnification 10-80×) to account for all parasitic copepods, including larvae and juveniles (Gee & Davey 1986). We then carefully removed the mussel tissue from the plates, deposited it in glass vials, froze (-20 °C for at least 24 hours) and freeze-dried it (48 hours) to ultimately measure the dry weight of the tissue. Condition index was determined as $CI = \frac{DW}{L^3}$, 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. Shell 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 environment R (R Development Core Team 2015) and model assumptions were confirmed using qq plots and histograms (Zuur et al. 2010). When data were not normally distributed, we applied appropriate transformations. $P$ values of $< 0.05$ were considered significant.
The condition index of wild mussels was log_{10}-transformed, before the difference in naturally infected and uninfected mussels was analysed with a Student’s t-test. The results of the two laboratory experiments were analysed separately, but using similar models. To test for effects of the parasite on clearance rate of the mussels, we applied a square-root transformation and used a linear mixed model (lmm; lmer function from the package lme4; Bates et al. 2015) with infection status, food level, time, the interaction between these three variables and experimental block as fixed factors. Individual mussels and successful filtration 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, with the number of *M. orientalis* individuals as predictor variable 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_{10}-transformed to improve normality of the data. To test for effects of the parasites on the condition of blue mussels, we applied a general linear model (glm) with infection status, food level, 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 with *M. orientalis* intensity.

Finally, to test for the effects of the parasites on mussel shell growth, we first modelled shell growth against mussel shell length at the start of the experiment with a linear model. We took the residuals from this model as a proxy for shell growth (corrected for initial length) and subsequently used a general linear model to test for the effects of infection status, food level, their interaction, and experimental blocking as explanatory variables. Finally, we tested the
effect of intensity of infection on (corrected) shell 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, n = 30, 30-50 mm*) in a mixed mussel (*M. edulis*) and Pacific oyster (*M. gigas*) bed on a tidal flat on the east coast of Texel was 53% with a mean (± SE) intensity of 1.8 (± 0.3) individuals per infected host. From this sample batch, 10 mussels had the same size class as the mussels used in our experiment (30-35 mm) and this group had a prevalence of 50% and mean intensity (± SE) of 3.0 ± 0.7. Naturally infected mussels (*n = 18*) tended to have 20% lower condition indices than uninfected mussels (*n = 21*), however the difference was not significant (*t = 8.880, *P* = 0.068; Figure 2).

[Figure 2 here]

**Success of controlled infections**

**Hatching success**

Dissection of 140 source mussels from a mixed blue mussel and Pacific oyster bed on the east coast of Texel (Vlakte van Kerken) produced 60 egg sacs (prevalence of gravid females was 43%). The time from egg sac extraction to hatching of copepod larvae was highly variable and ranged from immediate hatching to 8 d after extraction, with an average of 4.4 d. At early phases the eggs were opaque (Figure 3a), but when close to hatching, the eggs became transparent and the red eye spots of the larvae became visible through the egg case (Figure 3b). All eggs within
an individual egg sac developed at similar rates. The nauplius phase (Figure 3c) lasted a
maximum of 1 d, and infective copepodite I larvae (Figure 3d) appeared on average 4.8 d 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 (infective) stages were 240-290 µm long. An average of 50
copepodite larvae successfully emerged from a single female’s egg sac pair, although the
maximum recorded was more than 200 copepodites in a pair. Hatching success ranged from 0 to
100%, and overall, 26.1% of the eggs failed to hatch.

Infection success

In the first experiment, mussels had a higher infection success rate (88%) than in the second
experiment (76%). The maximum number of individual M. orientalis found in a single mussel
was 12. Average intensities of controlled infections in both experiments were comparable to
those of similar-sized mussels in the field (mean, SD; Experiment 1: 3.0, 2.4, Experiment 2: 3.5,
3.2). Like adults, juvenile copepods were found in the digestive tract near the stomach of blue
mussels and were approximately 2 mm long at termination of the experiment (Figure 4).

Therefore, the copepodites had increased about ten times in size, growing at a rate of about 30
µm day$^{-1}$ after infecting their hosts. As the copepods were not yet grown to mature size, it was
impossible to determine their sex. The uninfected control mussels were confirmed to be free of
infection, except for one mussel in the first experiment that was infected with two adult female
M. orientalis.
Effects on mussel clearance rate

No mortality occurred in either infected or uninfected mussels in either of the two experiments. In both experiments, 13% of the mussels did not filter during the experimental period, and non-filtering mussels occurred across all treatments. We did not find significant overall effects of infection status or food limitation on mussel clearance rates (Table SI), but, clearance rate significantly differed over time in the second (lmm; $P = 0.150 \times 10^{-13}$; Figure 5b) but not in the first experiment ($P = 0.722$; Figure 5a) 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 Experiment 2 in comparison to Experiment 1 (Figure 5). When testing for the effects of *M. orientalis* intensity upon infected mussels, we found no significant results for any of the factors in the first experiment and no significant interaction between food level and infection intensity, but a significant effect of time ($P = 0.261 \times 10^{-6}$; Table SI) in the second experiment.

Effects on mussel body condition

Experimentally infected mussels had significantly lower body condition indices compared to uninfected mussels (mean ± standard error, SE: Experiment 1: infected mussels 0.74 ± 0.03, uninfected mussels 0.81 ± 0.01; Experiment 2: infected mussels 0.68 ± 0.02, uninfected mussels 0.75 ± 0.02; Table SII; Figure 6). Furthermore, infected mussels kept under low food levels had the tendency to have lower condition indices, whereas uninfected mussels had slightly increased
condition indices (Figure 6). However, in both experiments, the effect of food level and the interaction between infection status and food level was not significant (Table SII).

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 experiments. In the first experiment, we found a positive relationship between *M. orientalis* intensity and body condition of mussels (*P* = 0.046), but this result was not significant in the second experiment (Table SII). Additionally, the block factor was significant in the first experiment (*P* = 0.013), but not significant in the second experiment (Table SII). Finally, we did not find any significant effects of food level or an interaction between intensity and food level in both experiments (Table SII).

[Figure 6 here]

**Effects on mussel shell growth**

Mean mussel shell growth (± SE) was 0.56 (± 0.05) mm in the first experiment and 0.49 (± 0.07) mm in the second experiment, which is an average of about 0.01 mm day\(^{-1}\). In both experiments, mussel shell growth (corrected for initial shell length) was not significantly affected by *M. orientalis* infection status, food level, the interaction between those variables or the blocking factor (Table SIII). Furthermore, among only the infected individuals in both experiments, 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 shell growth of infected mussels in either experiment (Table SIII).
This study experimentally tested for the effects of the invasive parasitic copepod *Mytilicola orientalis* (which has recently spilled over from invasive Pacific oysters) on native blue mussels. In laboratory experiments and in the field, we found significant negative effects of infection with (juvenile stages of) the invasive parasite on the body condition of mussels, although in the laboratory the feeding, growth, and survival were not affected.

The detrimental effect of early developmental stages of *Mytilicola* infection has been previously suggested for the congeneric *M. intestinalis* (Korringa 1950; Dethlefsen 1985) because of their presence in the ramifications of the digestive gland (Campbell 1970). This is the digestive organ in molluscs, and infections may compromise its functioning. As stable isotope analyses suggest that *Mytilicola* feeds on host tissue (Gresty & Quarmby 1991; Goedknegt et al. 2017b), the energy demand of the growing copepods can be expected to lead to a significant loss of tissue, ultimately resulting in a potential lower host condition (11-13% reduction in our experiments, but no detectable difference between infected and uninfected mussels in the wild).

Moreover, 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 & Davey 1986). A decrease in harmfulness with parasite age may also explain why we did not find significant difference in condition between infected and uninfected mussels in the field, as those infections consisted of a mix of juvenile and adult stages of *M. orientalis*. Generally, adverse effects of *M. orientalis* on host condition have also been reported for oysters, *Ostrea lurida* (Odlaug 1946 and *Magallana*...
**gigas** (Katkansky et al. 1967) and are known to increase with infection intensity (Katkansky et al. 1967). Similarly, for the congeneric species *M. intestinalis* reductions in dry weight of its blue mussel host are more severe when the parasite occurs in higher numbers in sympatric parasite-host populations (Feis et al. 2016). However, in our experiments we could not find a general trend of declining mussel condition with *M. orientalis* intensity, as the two experiments gave contrary results in this respect. Nevertheless, the general negative effect of infections with early stages of *M. orientalis* on mussel body condition suggests that native mussels may experience negative effects from the spillover of this invasive parasite. The exact mechanism behind the loss in body condition and the effect of the parasite on the energy budgets (lipids/glycogen content) of mussels, are a topic for future studies.

In contrast to the adverse effects of juvenile *M. orientalis* on mussel condition, we found no evidence that *M. orientalis* infection impacted the clearance rates of mussels. Previous work has 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 affect gill function in the same way as trematode metacercariae. Instead, *Mytilicola* infections may only indirectly affect filtration by influencing host energy requirements and expenditure. Alternatively, mussels could intensify their filter activity when infected with *M. orientalis* to counterbalance the higher energy demand caused by the parasite. However, we did not observe any significant effects of parasite infection on mussel clearance rates. We acknowledge that our inference in this respect might have been hampered due to the variation in clearance rates we observed over time, especially in the second experiment. Part of this variation is explained by the mussels that did not filter during our experiments, which we therefore 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\(^{-1}\), 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 may explain the limited shell growth of all mussels (on average 0.01 mm day\(^{-1}\)). The absence of any effect of *M. orientalis* on mussel shell growth may also be related to the low growth, but this result also corresponds with observational studies of Pacific oysters which did not detect negative effects of infections (Katkansky et al. 1967; Steele & Mulcahy 2001).

Finally, a negative effect of the parasite on mussel survival could have been expected given that its 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 experiments there was no mortality of mussels among 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 at the food levels applied which were chosen to lay within the range of concentration where mussels actually filter. It may well be that adverse effects of infections under even more extreme scenarios such as severe starvation may occur and future studies could investigate the effects of the parasite under such more extreme food conditions.

In general, controlled infections of hosts with *Mytilicola* infective larval stages proved to be an effective method to study effects of the parasite on blue mussel hosts, which can be applied in subsequent studies with other host species as they would 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 experiments, suggests that only sublethal effects of the parasite occurred, and that our experimental procedures were non-lethal. Furthermore, we have documented the maturation of *M. orientalis* larval stages from the moment of hatching to the development of the infective stage (see also Pogoda et al. 2012), 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 (about 3 copepods per infected host) that were similar to intensities observed in natural populations of similar sized infected mussels. As the results of our varying infection techniques only marginally differed between experiments, 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 & Davey (1986) 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* because of 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 our experimental study, we challenged the mussels with only 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 indirect parasite-mediated effects via parasite spillover.

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**References**


Gee JM, Davey JT. 1986. Stages in the life history of Mytilicola intestinalis STEUER, a copepod parasite of Mytilus edulis (L.), and the effect of temperature on their rates of development. ICES Journal of Marine Science 42:254–64.


Sampling locations of wild mussels (squares; n = 30 and n = 39), mussels as a source for gravid *Mytilicola orientalis* females (circles; n = 140) and naturally uninfected mussels (triangles; n = 150).
Condition index ($\log_{10}$-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 (*Magallana gigas*) bed on a tidal flat on the east coast of Texel (Vlakte van Kerken, Texel, The Netherlands). The boxes represent the interquartile range, the whiskers denote the lowest and highest values within the 1.5 interquartile range, the black line in each box denotes the median and the black dots represent the mean condition indices of each group. Note the truncated y-axis.

101x127mm (300 x 300 DPI)
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.
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.
Mean clearance rate (± SE) of uninfected blue mussels (grey) and mussels infected with *Mytilicola orientalis* (black) fed under high (circles) and low (triangles) food levels. Clearance rates were measured weekly after exposure to infective larvae for each of the four treatment groups in the first (a) and second (b) experiment.
Boxplots of the condition index (log_{10}-transformed) of mussels in the first (a) and second (b) experiment that were either infected (grey) or uninfected (white) and kept under high or low food level conditions during the nine weeks of the experiment. The boxes represent the interquartile range, the whiskers denote the lowest and highest values within the 1.5 interquartile range, the black line in each box denotes the median, the large black dots represent the mean condition indices of each group and the smaller dots outside the boxes are outliers. Note the truncated y-axis.
<p>| Data selection | Effect Factor | Experiment 1 | | | Experiment 2 | | |
|----------------|--------------|--------------|---|---|--------------|---|
| <strong>All mussels</strong> | Fixed Intercept | $\beta$ | 1.634 | 0.242 | 1.570 | 2.321 | 1.037 | 2.238 | 0.150 * $10^{-13}$ |
| | Fixed Food level | $\beta$ | -0.370 | 0.311 | -1.187 | 2.067 | 0.724 | 0.066 | 0.293 | 0.226 | 2.134 | 0.710 |
| | Fixed Infection status | $\beta$ | -0.073 | 0.312 | -0.233 | 0.566 | 0.967 | 0.024 | 0.293 | 0.081 | 2.502 | 0.664 |
| | Fixed Time | $\beta$ | -0.042 | 0.041 | -1.028 | 2.075 | 0.722 | -0.164 | 0.035 | -4.637 | 70.846 | |
| | Fixed Block | $\beta$ | 0.004 | 0.008 | 0.447 | 0.245 | 0.621 | -0.005 | 0.015 | -0.316 | 0.100 | 0.752 |
| | Fixed Food level * Infection status | $\beta$ | 0.187 | 0.441 | 0.424 | 0.467 | 0.792 | -0.081 | 0.414 | -0.195 | 1.938 | 0.380 |
| | Fixed Food level * Time | $\beta$ | 0.074 | 0.057 | 1.287 | 1.852 | 0.396 | 0.024 | 0.050 | 0.483 | 0.329 | 0.849 |
| | Fixed Infection * Time | $\beta$ | 0.016 | 0.057 | 0.279 | 0.391 | 0.822 | 0.008 | 0.050 | 0.163 | 0.421 | 0.810 |
| | Fixed Food level * Time * Infection status | $\beta$ | -0.048 | 0.081 | -0.594 | 0.352 | 0.553 | -0.040 | 0.071 | -0.560 | 0.313 | 0.576 |
| <strong>Random Variables</strong> | Var. | SD | | | Var. | SD | | |
| Individual mussels | 0.000 | 0.000 | | | 0.034 | 0.184 | | |
| Successful filtration | 2.053 | 1.433 | | | 1.728 | 1.314 | | |
| Residual | 0.540 | 0.735 | | | 0.747 | 0.864 | | |
| <strong>Infected mussels only</strong> | Fixed Intercept | $\beta$ | 1.854 | 1.071 | 1.730 | 1.297 | 1.260 | 1.030 | |
| | Fixed Infection intensity | $\beta$ | -0.076 | 0.069 | -1.093 | 2.834 | 0.586 | 0.063 | 0.120 | 0.525 | 6.254 | 0.181 |
| | Fixed Food level | $\beta$ | -0.765 | 0.554 | -1.381 | 4.040 | 0.401 | 0.150 | 0.457 | 0.328 | 6.290 | 0.179 |
| | Fixed Time | $\beta$ | -0.098 | 0.061 | -1.594 | 3.681 | 0.451 | -0.208 | 0.057 | -3.666 | 36.219 | 0.261 * $10^{-6}$ |
| | Fixed Block | $\beta$ | 0.010 | 0.013 | 0.760 | 0.702 | 0.402 | 0.026 | 0.025 | 1.053 | 1.077 | 0.299 |
| | Fixed Food level * Time | $\beta$ | 0.181 | 0.101 | 1.795 | 3.383 | 0.184 | 0.087 | 0.075 | 1.155 | 1.496 | 0.473 |
| | Fixed Infection intensity * Time | $\beta$ | 0.015 | 0.013 | 1.212 | 2.600 | 0.458 | 0.020 | 0.020 | 1.007 | 5.445 | 0.142 |
| | Fixed Infection intensity * Food level | $\beta$ | 0.116 | 0.146 | 0.793 | 2.117 | 0.347 | -0.043 | 0.136 | -0.320 | 5.444 | 0.066 |
| | Fixed Food level * Time * Infection intensity | $\beta$ | -0.033 | 0.027 | -1.226 | 4.288 | 0.368 | -0.025 | 0.022 | -1.138 | 5.656 | 0.226 |
| <strong>Random Variables</strong> | Var. | SD | | | Var. | SD | | |
| Individual mussels | 0.002 | 0.050 | | | 0.051 | 0.227 | | |
| Successful filtration | 2.041 | 1.429 | | | 1.947 | 1.395 | | |
| Residual | 0.518 | 0.720 | | | 0.703 | 0.839 | | |</p>
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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 (*Magallana gigas*) co-introduced the parasitic copepod *Mytilicola orientalis* that spills over to native blue mussels (*Mytilus edulis*) and other native bivalves. In this study, we investigated the impact of *M. orientalis* infections on blue mussels by conducting laboratory experiments 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 a significantly lower body condition (11–13%) than uninfected mussels after nine weeks of infection. However, naturally infected mussels from a mixed mussel and oyster bed did not significantly differ in body condition than uninfected mussels. Contrary to effects on mussel condition, we did not find an effect of experimental infections on clearance rates, shell 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. The results of this study call for the integration of such parasite-mediated indirect effects of invasions in impact assessments of invasive species.

Keywords

controlled infection, Magallana gigas, invasive species, Pacific oyster, parasite co-introduction, parasite spillover
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, *Magallana* (previously *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 digestive tract 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 wild native blue mussels
(Mytilus edulis Linnaeus, 1758), common cockles (Cerastoderma edule Linnaeus, 1758), Baltic
tellins (Limecola (formerly Macoma) balthica Linnaeus, 1758) and European flat oysters (Ostrea
edulis Linnaeus, 1758), indicating several spillover events (His 1977; Stock 1993; Pogoda et al.
2012; Goedknegt et al. 2017). In particular, native blue mussels 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 described from the
Mediterranean Sea (Steuer, 1902), which was for the first time found in the Wadden Sea in 1938
(Caspers 1939) and that has a similar direct life cycle as M. orientalis (Caspers 1939; Grainger
1951; Dethlefsen 1985; Gee & Davey 1986). Mytilicola intestinalis became infamous as the ‘red
worm disease’, because it was thought 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 to support this hypothesis is rare. Historically, especially juvenile
stages of M. intestinalis were held responsible for mortalities of mussels (Korringa 1950;
Dethlefsen 1985) because of 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 their egg yolk, the only food source available during the pelagic larval phase
(Grainger 1951). This increases the chance for mussels to become negatively affected by
juvenile parasites. For mature M. intestinalis, lethal and sublethal effects on important host
fitness components such as body condition, filtration rate and reproduction do not seem to be
one-directional and have been controversially discussed (Lauckner 1983). Only recently,
controlled infection experiments have been conducted with M. intestinalis and blue mussels in
which a reduction in blue mussel dry weight was found as a result of infection by the parasite in sympatric parasite-host populations (Feis et al. 2016). Similarly, although *M. orientalis* is generally considered a serious pest (Holmes and Minchin 1995) and is registered on the list of 100 worst invaders of the Mediterranean (Streftaris and Zenetos 2006), studies on its lethal and sublethal effects on *M. gigas* (Katkansky et al. 1967; Deslous-Paoli 1981; De Grave et al. 1995; Steele & Mulcahy 2001) and *Ostrea lurida* Carpenter, 1864 (Odlaug 1946) have been inconclusive and they have generally lacked experimental approaches. The effects of *M. orientalis* on its new host, the blue mussel, have not been studied to date. A potential effect of *M. orientalis* on native blue mussels is likely to be modified by environmental factors and species interactions (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 the 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 effect of *Mytilicola* spp. infections on hosts at low food levels is lacking.

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

**Material and Methods**

**Field survey**

To determine natural prevalence (the proportion of infected individuals) and mean infection intensity (mean number of parasites in infected mussels) of *Mytilicola orientalis* in blue mussels in the wild, we collected 30 mussels of 30-50 mm length from a mixed bed of Pacific oysters and blue mussels located on the Vlakte van Kerken, a tidal flat on the east coast of the island of Texel (Figure 1) in the western European Wadden Sea (southern North Sea) on 11 June 2014.

Additionally, we collected from this bed another 39 mussels (34-54 mm) to analyse for the effect of infection status (infected/uninfected) on mussel body condition. For methods on the assessments of infection status and condition index see section Body condition and shell growth.

[Figure 1 here]

**Experimental infections**

Uninfected mussels (30-35 mm; *n* = 150) for the experiments were haphazardly collected from basalt groins on the north-west shore of the Dutch mainland (Julianadorp, Figure 1) on 11 September 2014. Previous explorations by M. A. Goedknegt had shown that *Mytilicola* spp. does not occur at this location, which was verified by haphazardly sampling and screening 30 additional mussels, all of which turned out were all found to be not infected with *Mytilicola free of infection*. Any epifauna (mostly barnacles) on the mussels was carefully removed from the...
shells to ensure that copepod larvae could infect mussels without being eaten or physically obstructed during experimental infections (Johnson & 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 Parke, 1949 culture, or alternatively with Phyto-Feast® when fresh culture was unavailable (on average once a week).

To acquire a source for *M. orientalis* larvae, mussels (*n* = 140) were haphazardly collected from a mixed bed with known *Mytilicola* infections located on a tidal flat on the east coast of the island of Texel (Figure 1) on 22 August 2014. Within two days of collection, mussels were dissected and gravid *M. orientalis* 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 & Davey (1986). When larvae had developed into the infective copepod I stage (Gee & Davey 1986), uninfected mussels (*n* = 74) were exposed to the larvae.

Before exposure to infective larvae, the shell length (maximum anteroposterior axis) of each mussel was measured with callipers to the nearest mm. Because of variation in larval hatching and development time, insufficient larvae were available to infect all mussels at once, ultimately resulting in two temporal experimental batches of infected mussels that formed the base of the experiment (see section Experimental set up for sample sizes). These two temporal batches also allowed us to experiment with two infection protocols, because this was the first
time that artificial infections were conducted with *M. orientalis*. Individual mussels in the first batch of the experiments of infected mussels. Individual mussels in the first experiment (*n* = 34 mussels) were exposed to parasites in a 100-mL cup and after 24 h, mussels and filtered sea water were transferred to a 1000-mL container for the following eight weeks of the experiment. For individual mussels in the second experimental batch experiment (*n* = 30), 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 infective 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 filtered sea water. To promote filtration and uptake of infective larvae, small amounts of algal culture (*I. galbana*) were added to the sea water during exposure of mussels from batch two of the second experiment. Five extra mussels per each of the two batches experiments were artificially infected and sacrificed for examination of larval development at mid-way points during the experiments. Control (uninfected) mussels (Batch Experiment 1: *n* = 34, Batch Experiment 2: *n* = 30) were treated identically to exposed mussels within each batch experiment (transfer of filtered sea water and small amounts of algal culture) but without the addition of copepodites.

**Experimental set-up**

The experiment was set up immediately after exposing the mussels to the parasite larvae, which was for the first batch experiment on 10 September 2014 and the second batch experiment on 7 October 2014. 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 each containing one individual mussel. Thus, each block contained a replicate of
the following treatments: uninfected mussel – low food level, infected mussel – low food level,
uninfected mussel – low high food level, and infected mussel – high food level. The first batch of
the experiment contained 17 of these replicated blocks (n = 68 mussels in total), while the second
batch experiment contained 15 replicated blocks (n = 60 mussels in total). 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 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, on average once per week, 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. On
average only fresh algal culture was unavailable once a week, but never provided on days when
clearance rates were measured.

After dissections at the end of the experiment, we found that some experimental blocks
contained 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). In these cases, affected blocks were excluded from the analysis to preserve a
balanced design with a complete dataset. In the first batch of the experiment, 4 four mussels (out
of 34 mussels exposed to larvae) remained uninfected and one mussel was unintentionally
infected, while in the second batch experiment infection success was lower and 7 seven (out of
30) mussels where left were uninfected. After removing all blocks with failed and unanticipated
infections, 12 blocks were left for the first batch experiment (n = 48 mussels) and 10 blocks for
the second batch experiment (n = 40 mussels) of the experiment. 

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Measurement of clearance rate, body condition and shell growth

Clearance rate

We conducted the first clearance rate measurements of the first batch of mussels of the first experiment one week after exposure to *M. orientalis* larvae, while the clearance rate of mussels of the second batch of mussels experiment 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 eight (Batch 1) and nine weeks (Batch 2) and immediately after termination of the respective batch all mussels were measured, screened for presence of *M. orientalis* larvae, frozen, freeze-dried and weighed to assess body condition index (see next section Body condition and shell growth).

Clearance rate was assessed by means of the indirect clearance method (Riisgård 2001; Petersen et al. 2004). One day prior to the measurement, all containers were refreshed with filtered seawater and mussels were checked for survival (severely gaping mussels in smelly water were assigned as dead). 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 Relative Fluorescence Units (RFU) using a Trilogy® Laboratory Fluorometer (Turner Designs). During the test, algal concentration measurements were performed on the fluorometer as its high measurement rate, which 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 created 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 by 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. 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 shell growth**

At the experiments ran for eight (Experiment 1) and nine weeks (Experiment 2) and immediately after termination of the experiment, we dissected experiments all mussels to check were measured, screened for the presence of *M. orientalis* in “infected” mussels and absence in the “control” mussels larvae, frozen, freeze-dried and weighed to assess body condition. 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. Mussel tissue was compressed between glass slides and examined under a stereo microscope (magnification 10-80×) to account for all parasitic copepods, including larvae and juveniles (Gee & Davey 1986). We then carefully removed the mussel tissue from the plates, deposited it in glass vials, froze (-20 °C for at least 24 hours) and freeze-dried it (48 hours) to ultimately measure the dry weight of the tissue. 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. Shell 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 environment R (R Development Core Team 2015) and model assumptions were confirmed using qq plots and histograms (Zuur et al. 2010). When data were not normally distributed, we applied appropriate transformations. *P* values of < 0.05 were considered significant.

The condition index of wild mussels was log₁₀-transformed, before the difference in naturally infected and uninfected mussels was analysed with a Student’s t-test. Regarding the laboratory experiment, each The results of the two experimental batches was laboratory experiments were analysed separately, but using 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 and used a linear mixed model (lmm; lmer function from the package lme4; Bates et al. 2015) with infection status, food level, time, the interaction between these three variables
and experimental block as fixed factors. Individual mussels and successful filtration 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 we used the number of *M. orientalis* individuals as predictor variable 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$_{10}$-transformed to improve normality of the data. To test for effects of the parasites on the condition of blue mussels, we applied a general linear model (glm) with infection status, food level, 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 with *M. orientalis* intensity.

Finally, to test for the effects of the parasites on mussel shell growth, we first modelled shell growth against mussel shell length at the start of the experiment with a linear model. We took the residuals from this model as a proxy for shell growth (corrected for initial length) and subsequently used another general linear model to test for the effects of infection status, food level, their interaction, and experimental blocking as explanatory variables. Finally, we tested the effect of intensity of infection on (corrected) shell 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, n = 30, 30-50 mm) in a mixed mussel (M. edulis) and Pacific oyster (M. gigas) bed on a tidal flat on the east coast of Texel was 53% with a mean (± SE) intensity of 1.8 (± 0.3) individuals per infected host. From this sample batch, 10 mussels had the same size class as the mussels used in our experiment (30-35 mm) and this group had a prevalence of 50% and mean intensity (± SE) of 3.0 ± 0.7. Naturally infected mussels (n = 18) tended to have 20% lower condition indices than uninfected mussels (n = 21), however the difference was not significant (t = 8.880, P = 0.068; Figure 2).

Success of controlled infections

Hatching success

Dissection of 140 source mussels from a mixed blue mussel and Pacific oyster bed on the east coast of Texel (Vlakte van Kerken) produced 60 egg sacs (prevalence of gravid females was 43%). The time from egg sac extraction to hatching of copepod larvae was highly variable and ranged from immediate hatching to 8 days after extraction, with an average of 4.4 d. At early phases the eggs were opaque (Figure 3a), but when close to hatching, the eggs became transparent and the red eye spots of the larvae became visible through the egg case (Figure 3b). All eggs within an individual egg sac developed at similar rates, however hatching success ranged from 0 to 100%, and 26.1% of the eggs failed to hatch. The nauplius phase (Figure 3c) lasted a maximum of 1 d, and infective copepodite I larvae (Figure 3d) appeared on average 4.8 d 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...
220 µm in length and the copepodite I (infective) stages were 240-290 µm long. An average of 50 copepodite larvae successfully emerged from a single female’s egg sac pair, although the maximum recorded was more than 200 copepodites in a pair. Hatching success ranged from 0 to 100%, and overall, 26.1% of the eggs failed to hatch.

Infection success

In the first batch of experiment, mussels had a higher infection success rate (88%) than in the second batch experiment (76%). The maximum number of individual *M. orientalis* found in a single mussel was 12. Average intensities of controlled infections in both batches experiments were comparable to those of similar-sized mussels in the field (mean, SD; Batch Experiment 1: 3.0, 2.4, Batch Experiment 2: 3.5, 3.2). Like adults, juvenile copepods were found in the digestive tract near the stomach of blue mussels and were approximately 2 mm long at termination of the experiment (8 or 9 weeks; Figure 4). Therefore, the copepodites had increased about ten times in size, growing at a rate of about 30 µm day\(^{-1}\) since they infected their hosts. As the copepodites were not yet grown to mature size, it was impossible to determine their sex. The uninfected control mussels were confirmed to be free of infection, except for one mussel in the first batch experiment that was infected with two adult female *M. orientalis*.

Effects on mussel clearance rate
During the entire experimental period, no mortality occurred in either infected and/or uninfected mussels in either of the two batch experiments. In both batch experiments, 13% of the mussels did not filter during the experimental period, and non-filtering mussels occurred across all treatments. For either batch in this experiment we did not find significant overall effects of infection status or food limitation on mussel clearance rates (Table I, Figure 5). However, clearance rate significantly differed over time in the second (Imm; $P = 0.150 \times 10^{-13}$; Figure 5b, Table I) but not in the first batch experiment ($P = 0.722$; Figure 5a, Table I) 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 experiment 2 in comparison to batch experiment 1 of the experiment (Figure 5). When testing for the effects of *M. orientalis* intensity upon infected mussels, we found no significant results for any of the factors in the first batch experiment and no significant interaction between food level and infection intensity, but a significant effect of time ($P = 0.261 \times 10^{-6}$; Table ISI) in the second batch of the experiment. 

**Effects on mussel body condition**

Experimentally infected mussels had significantly lower body condition indices compared to uninfected mussels (mean ± standard error, SE: Batch Experiment 1: infected mussels 0.74 ± 0.03, uninfected mussels 0.81 ± 0.01; Batch Experiment 2: infected mussels 0.68 ± 0.02, uninfected mussels 0.75 ± 0.02; Table II; Figure 6). Furthermore, infected mussels kept under low food levels had the tendency to have lower condition indices, whereas uninfected mussels had slightly increased condition indices (Figure 6). However, in both
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 batch experiments. In the first batch experiment, we found a positive relationship between *M. orientalis* intensity and body condition of mussels (*P* = 0.046), but this result was not significant in the second experimental batch experiment (Table SII). Additionally, the block factor was significant in the first batch experiment (*P* = 0.013), but not significant in the second batch of the experiment. (Table SII). 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 SII).

**Effects on mussel shell growth**

Mean mussel shell growth (± SE) was 0.56 (± 0.05) mm in the first batch experiment 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 batch experiments, mussel shell growth (corrected for initial shell length) was not significantly affected by *M. orientalis* infection status, food level, the interaction between those variables or the blocking factor (Table SIII). Furthermore, among only the infected individuals in both batch experiments, 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 shell growth of infected mussels in either batch experiment (Table SIII).
This study experimentally tested for the effects of the invasive parasitic copepod *Mytilicola orientalis* (which has recently spilled over from invasive Pacific oysters) on native blue mussels. By using controlled infections and in laboratory experiments and in the field, we found significant negative effects of infection with (juvenile stages of) the invasive parasite on the body condition of mussels. Naturally infected mussels from the field also tended to have a lower body condition than uninfected mussels, but the difference was not significant. We did not find evidence for parasite effects on mussel clearance rates, survival or shell growth, although in the laboratory the feeding, growth, and survival were not affected.

The detrimental effect of early developmental stages of *Mytilicola* infection has been previously suggested for the congeneric *M. intestinalis* (Korringa 1950; Dethlefsen 1985) because of their presence in the ramifications of the digestive gland (Campbell 1970). This is the digestive organ in molluscs, and infections may compromise its functioning. As stable isotope analyses suggest that *Mytilicola* feeds on host tissue (Gresty & Quarmby 1991; Goedknegt et al. In press.), the energy demand of the growing copepods can be expected to lead to a significant loss of tissue, ultimately resulting in a potential lower host condition (11-13% reduction in our experiments, but no statistically significant difference between infected and uninfected mussels in the wild). 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 & Davey 1986). A decrease in harmfulness with parasite age may also explain why we found no significant difference in condition between infected and uninfected mussels in the field, as those infections consisted of a mix of juvenile and adult stages of *M. orientalis*. Generally, adverse effects of *M. orientalis* on host condition have also been reported for oysters, *Ostrea lurida* (Odlaug 1946 and *Magallana gigas* (Katkansky et al. 1967) and are known to increase with infection intensity (Katkansky et al. 1967). Similarly, for the congeneric species *M. intestinalis* reductions in dry weight of its blue mussel host are more severe when the parasite occurs in higher numbers in sympatric parasite-host populations (Feis et al. 2016). However, in our experiments we could not find a general trend of declining mussel condition with *M. orientalis* intensity, as the two experimental batches gave contrary results in this respect. Nevertheless, the general negative effect of infections with early stages of *M. orientalis* on mussel body condition suggests that native mussels may experience negative effects from the spillover of this invasive parasite. The exact mechanism behind the loss in body condition and the effect of the parasite on the energy budgets (lipids/glycogen content) of mussels, are a topic for future studies.

In contrast to the adverse effects of juvenile *M. orientalis* on mussel condition, we found no evidence for an effect of that *M. orientalis* infection on the 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 affect gill function in the same way as trematode metacercariae. Instead, *Mytilicola* infections may only indirectly affect filtration by influencing host energy requirements and expenditure. Alternatively, mussels could intensify their filter...
activity when infected with *M. orientalis* to counterbalance the higher energy demand caused by the parasite. However, we did not observe any significant effects of parasite infection on mussel clearance rates. We acknowledge that our inference in this respect might have been hampered due to the variation in clearance rates we observed 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 experiments, which we therefore 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\(^{-1}\), 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 may explain the limited shell growth of all mussels (on average 0.01 mm day\(^{-1}\)). The absence of any effect of *M. orientalis* on mussel shell growth may also be related to the low growth, but this result also corresponds with observational studies of Pacific oysters which did not detect negative effects of infections (Katkansky et al. 1967; Steele & Mulcahy 2001). Finally, a negative effect of the parasite on mussel survival could have been expected given that its 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 experiments 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 at the food levels applied which were chosen to lay within the range of concentration where mussels actually filter (see Material and Methods section). It may well be that adverse effects of infections under even more extreme scenarios
such as severe starvation may occur and future studies could investigate the effects of the parasite under such more extreme food conditions.

In general, controlled infections of hosts with *Mytilicola* infective larval stages proved to be an effective method to study effects of the parasite on blue mussel hosts, which can be applied in subsequent studies with other host species as they would 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 experiments, suggests that only sublethal effects of the parasite occurred, and that our experimental procedures were non-lethal. Furthermore, we have documented the maturation of *M. orientalis* larval stages from the moment of hatching to the development of the infective stage (see also Pogoda et al. 2012), 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 (about 3 copepods per infected host) that were similar to intensities observed in natural populations of similar sized 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 & Davey (1986) 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_ because of 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 with only 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 indirect parasite-mediated effects via parasite spillover.

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doi:10.1016/j.seares.2015.12.003


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doi:10.3354/meps211275


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doi:10.3354/meps11250


doi:10.12681/mms.180

Table I 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 first model included the effects on all mussels, while the second 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. Significant variables are stated in bold.

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Table II. Results of linear models that tested for effects of infection with *Mytilicola orientalis* and food level on blue mussel condition (log$_{10}$-transformed) in the two batches of the experiment. The effects of infection status and food level were tested for all mussels, while the effect of infection intensity was only tested for infected mussels in the experiment. Significant variables are stated in bold.

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Table III. Results of linear models that tested for effects of infection with *Mytilicola orientalis* and food level on blue mussel shell 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 *M. orientalis* intensity tested was only tested for infected mussels in the experiment.

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