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Trophic relationship between the invasive parasitic copepod *Mytilicola orientalis* and its native blue mussel (*Mytilus edulis*) host

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Abstract

Invasive parasites can spill over to new hosts in invaded ecosystems with often unpredictable trophic relationships within the new associations. In European seas, the intestinal copepod *Mytilicola orientalis* was co-introduced with Pacific oysters (*Crassostrea gigas*) and spilled over to native blue mussels (*Mytilus edulis*). This intestinal parasite caused a reduction in the condition of the mussels. However, whether the parasite feeds on host tissue and/or stomach contents is yet unknown. To answer this question, we performed a stable isotope analysis in which we included host tissue and the primary food sources of the mussels, microphytobenthos (MPB) and particulate organic matter (POM). The copepods were significantly enriched in ^{15}N and ^{13}C ratios relative to their mussel hosts (mean ± SD; 1.22 ± 0.58‰ for $\delta^{15}N$, 0.25 ± 0.32‰ for $\delta^{13}C$). Mixing models indicated that, in addition to host tissue (probabilities of 25 - 89%), also POM (8 - 35%) and MPB (1 - 45%) made up substantial proportions of the parasites' diet. These results suggested that the copepods are parasitic as well as commensalistic. This study illustrates the usefulness of isotope studies to unravel trophic relationships in new parasite-host associations in the course of invasions.

Introduction

Predation and parasitism are important trophic interactions that shape ecological communities and food webs. The key differences between predators and parasites are their relative size compared to their victims (parasite < host and predator > prey) and the number of victims made during a life-history stage (one for a parasite, but more than one for a predator; Lafferty and Kuris, 2002). Additionally, in food webs, predators practically always have a higher trophic position than their prey, while the trophic position of parasites can be more complex. Firstly, parasites with complex life cycles involving multiple hosts may feed on different trophic levels across distinct life cycle stages, making it difficult to determine a single trophic level for all parasite life cycle stages (Lafferty et al., 2008). Secondly, some parasites feed on various host tissues and some may not feed directly on the host at all, but rather on the host' stomach contents or specific pre-digested biochemical compounds (Iken, 2001; Lafferty et al., 2008). Hence, some endoparasites living inside a host's intestine may not necessarily be true parasites living strictly on host tissue, but may rather live in a (partial) commensal relationship with their host.

To address the latter problem, traditionally an analysis of parasite stomach contents was used to confirm a parasite-host relationship, but recently stable isotope analysis (SIA) has been proven to be a valuable method to determine the trophic position of parasites (e.g., Pinnegar et al., 2001; Deudero et al., 2002) and other consumers (e.g., Inger et al., 2006; Dubois et al., 2007). This method uses the differences (Δ) between isotopic ratios of naturally occurring stable isotopes of nitrogen ($\delta^{15}N$) and carbon ($\delta^{13}C$) between consumers and their diet to reconstruct trophic relationships (Post, 2002). The $\delta^{13}C$ discrimination factor ($\Delta^{13}C$) is used to determine the diet source of carbon (e.g., terrestrial vs marine primary producers; Hobson, 1986), while trophic enrichment ($\Delta^{15}N$) is used to estimate the trophic position (Vander Zanden et al., 1997), in which a fixed value (also known as the trophic fractionation factor) of 3.4‰ is most commonly used to analyse relative species trophic levels (Minagawa and Wada, 1984; Vander Zanden et al., 1997; Post, 2002).

In this study, we analyse the trophic relationship between the invasive endoparasite *Mytilicola orientalis* and its new host in European seas, the native blue mussel *Mytilus edulis* in the Dutch Wadden Sea. This parasitic copepod has been recently co-introduced with imports of the invasive Pacific oyster *Crassostrea gigas* (Elsner et al., 2011) and is known to spill over to native bivalves such as blue mussels and to a lesser extent to common cockles (*Cerastoderma edulis*) and Baltic tellins (*Limecola* (formerly *Macoma*) *balthica*; Goedknegt et al., 2017). *M orientalis* was first described in the Sea of Japan (Mori, 1935) and has a direct life cycle with a short non-feeding free-living stage, after which it lives in the intestines of its host. Here, the parasite is either feeding directly on the host tissue or indirectly on host gut content, resulting in a reduction in body condition of infected blue mussels (Chapter 7). As the exact diet source of the parasite is yet unknown, we performed a SIA to clarify the trophic relationship between the parasite *M. orientalis* and its new blue mussel host. Field samples of mussel hosts and parasites were analysed as well as the two principal food sources of mussels, being particulate organic matter and microphytobenthos (Dubois et al., 2007). This approach allowed us to determine the relative contributions of host tissue and host food to the diet of the invasive copepod and to identify the trophic relationship of this new parasite-host association that has resulted from the recent co-introduction of the copepods with their oyster hosts.

Material and methods

Collection of samples

Suspended particulate organic matter (POM) samples (n = 17) were collected on the 2nd and 4th of July 2013 at nine locations in the subtidal Marsdiep channel (Wadden Sea, The Netherlands, Fig. 8.1). At high tide, water from this channel feeds a small intertidal bay in the south of the island of Texel (Mok, The Netherlands) and therefore we assumed that POM originating from this channel is a major food source for blue mussels (*Mytilus edulis*) living in this bay where we sourced the mussels and parasites for the SIA (Fig. 8.1; see below). At each sampling point, water samples were collected with a Niskin bottle from approximately 1 m below the water surface. Samples were then sieved through a 200 µm mesh to exclude larger zooplankton from the sample and subsequently filtered onto pre-combusted 25 mm GF/F filters using a 25 mm filter cartridge mounted on a 60 mL syringe. Between 80 and 250 mL of water was filtered depending on the amount of suspended matter in the water column. Filters were then stored at -20 °C until further analysis.

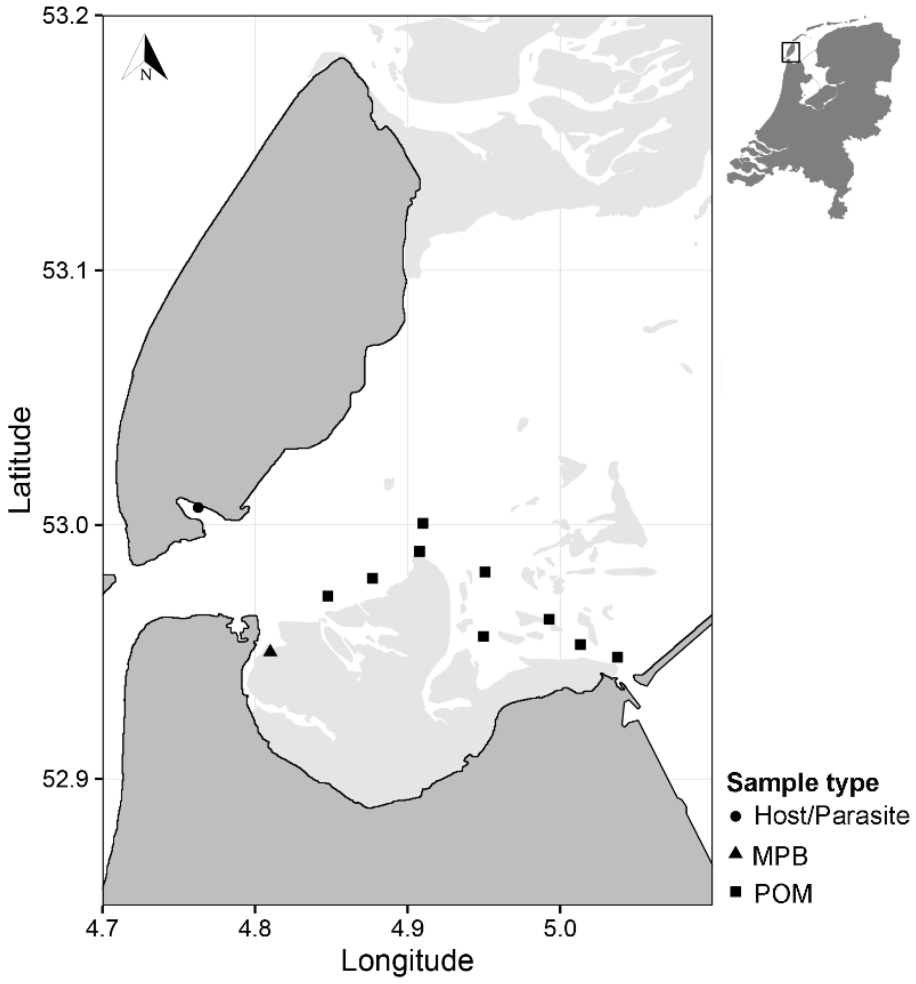

Fig. 8.1 Sampling locations of suspended particulate organic matter (POM), microphytobenthos (MPB), hosts (*Mytilus edulis*) and parasites (*Mytilicola orientalis*). On the microphytobenthos location, four samples were taken in an area of approximately 50 m^2. POM locations were sampled once (2 locations), twice (6 locations) or three times (1 location), adding to a total of 17 samples.

Microphytobenthos (MPB; n = 4 samples within an area of 50 m^2; Fig. 8.1) was sampled in the beginning of July 2013 at an intertidal area south of the Marsdiep (Wadden Sea, The Netherlands, Fig. 8.1) by collecting sediment from diatom mats into plastic bottles that were put on ice and brought to the research facility. Here, the sediment was spread in a tray, covered by three layers of nylon mesh (2 x 100 µm, 1 x 50µm) that was kept moist by repeatedly spraying filtered seawater on top. The samples were then left in a temperature-regulated room overnight at 20°C. The next morning, the algae were washed into a beaker with filtered seawater. This solution was centrifuged (10 min at 1000x g) and the remaining pellet was collected and stored at -20 °C.

Blue mussel and parasite (*Mytilicola orientalis*) samples were collected about three months later than the POM and MPB samples (26 September 2013), to cover the minimum time it takes for the diet to be incorporated into consumer tissue (Dubois et al., 2007; Phillips et al., 2014). Mussels (n = 150) were collected from a mixed oyster and mussel bed located in the Mok (Fig. 8.1) and checked for presence of *M. orientalis* parasites under a magnification glass (magnification 3 - 8×). Mussels infected with at least two parasites (including at least one big female; n = 28 mussels) were selected for the analysis, as a minimum of 0.4 mg dry weight of each pooled parasite and corresponding mussel sample (the adductor muscle of the mussel) were required for the SIA. Both parasite and matched mussel samples (each n = 28) were then stored at -20 °C.

Stable Isotope Analysis (SIA)

Prior to the SIA, all samples were freeze-dried for 48 hours at -60 °C to remove water content. Additionally, as *M. orientalis* is a crustacean, parasite samples were treated with 1 M HCl to remove inorganic carbonate and dried for another 24 h at 60 °C. Isotope ratios of $\delta^{15}N$ and $\delta^{13}C$ in all samples were determined with a Thermo Scientific Delta V Advantage Isotope Ratio Mass Spectrometer equipped with a Flash 2000 Organic Element Analyser at the Royal Netherlands Institute for Sea Research, Texel, The Netherlands. In addition, mean total nitrogen (TN) and total organic carbon content (TOC) were determined for hosts and parasites, but due to logistic constraints this was not possible for the POM and MPB samples.

The standard reference materials Acetanilide (SD: $\delta^{15}N$ 0.3‰, $\delta^{13}C$ 0.1‰) and Urea were respectively used as a correction and control of the isotope ratios found in the samples. Isotope ratios of $\delta^{15}N$ and $\delta^{13}C$ were then expressed as permille (‰) differences from a standard reference material using the formula $X = (R_{sample}/R_{standard} - 1) * 1000$, with R being the ratio between the heavy and light isotopes of nitrogen (^{15}N:^{14}N) and carbon (^{13}C:^{12}C). The reference material used for ^{15}N was atmospheric nitrogen N_2 and for ^{13}C Vienna Peedee-Belemnite Limestone (vPDB).

Statistical analysis

Normality and homoscedasticity of the data were checked with histograms, qqplots and boxplots (Zuur et al., 2010). Subsequently, differences in isotope ratios ($\delta^{13}C$ and $\delta^{15}N$) among the trophic groups (POM, MPB, hosts, parasites) were analysed with ANOVA's and post-hoc Tukey tests. Furthermore, comparisons and relationships between stable isotope data of parasites and corresponding hosts ($\Delta^{13}C$ and $\Delta^{15}N$) were made using paired Student's t-tests and Pearson correlations, respectively. All statistical analyses were performed with the statistical software program R (R Development Core Team, 2015).

Mixing models

The relative contribution of diet sources in the consumers' diet can be determined by the use of stable isotope mixing models (Phillips et al., 2003; Inger et al., 2006). In this study, we used a mixing model to determine the relative contributions of host tissue (blue mussel) and host gut content (POM, MPB) to the diet of the parasitic copepod *M. orientalis*. The package simmr (Parnell, 2016) was used to solve mixing equations for stable isotopic data within a Bayesian framework in the software program R (R Development Core Team, 2015). This package allows the use of multiple diet sources and with adjustable source specific trophic fractionation factors. In the first mixing model, individual $\delta^{15}N$ and $\delta^{13}C$ values of the parasite samples were used as the consumer data. Diet source data included the mean (± SD) $\delta^{15}N$ and $\delta^{13}C$ values of the sources POM, MPB and blue mussel, and were corrected for trophic fractionation. Here, we used the standard trophic fractionation factors of 3.4‰ for $\delta^{15}N$ and 1.0‰ for $\delta^{13}C$ for all diet sources (Minagawa and Wada, 1984; Vander Zanden et al., 1997; Post, 2002), as controlled diet studies and thus taxon-specific fractionation factors are not (yet) available for the parasite. However, $\delta^{15}N$ is known to scale negatively with trophic level (Caut et al., 2009; Hussey et al., 2014) and since the blue mussel is one trophic level higher than the other diet sources (POM and MPB), we carried out a sensitivity analysis for the fractionation factor for the blue mussel. We varied the values used for $\delta^{15}N$ between 1 and 4‰ to determine how much the estimated relative contribution of all diet sources changed with the fractionation factor.

Results

All trophic groups

Trophic groups differed significantly in $\delta^{15}N$ (ANOVA; $F_{3,73} = 588.16$, $p < 0.001$) and $\delta^{13}C$ ($F_{3,73} = 200.41$, $p < 0.001$). Values of $\delta^{15}N$ were highest for the parasitic copepod and lowest for POM, while for $\delta^{13}C$ MPB and POM had the highest and lowest values, respectively (Table 8.1; Fig. 8.2).

Table 8.1 Mean values (± SE) of $\delta^{15}N$ (‰) and $\delta^{13}C$ (‰) for suspended particulate organic matter (POM), microphytobenthos (MPB), the blue mussel host (*Mytilus edulis*) and its intestinal parasite *Mytilicola orientalis*.

Value	POM (n = 17)	MPB (n = 4)	Host *Mytilus edulis* (n = 28)	Parasite *Mytilicola orientalis* (n = 28)
$\delta^{15}N$ (‰)	5.97 ± 0.21	9.44 ± 0.18	11.42 ± 0.07	12.64 ± 0.07
$\delta^{13}C$ (‰)	-21.86 ± 0.28	-15.67 ± 0.09	-18.08 ± 0.06	-17.83 ± 0.07
TN (%)	NA	NA	7.99 ± 0.17	7.76 ± 0.18
TOC (%)	NA	NA	35.92 ± 0.69	35.18 ± 1.02

Parasites and hosts

Parasitic copepods were significantly enriched in ^{15}N and ^{13}C ratios relative to their host, the blue mussel (Student's paired t-test; $\delta^{15}N$: $t = 11.178$, $df = 27$, $p < 0.0001$; $\delta^{13}C$: $t = 4.0714$, $df = 27$, $p < 0.001$; for means see Table 8.1). However, the level of enrichment was relatively small (mean ± SD; 1.22 ± 0.58‰ for $\delta^{15}N$ and 0.25 ± 0.32‰ for $\delta^{13}C$). This minor enrichment of the parasite in relation to its host was not reflected in the differences in mean total nitrogen (TN) and total

organic carbon content (TOC) in both tissues (Student's t-test; TN (%): t = -1.361, df = 27, p = 0.185; TOC (%): t = -0.741, df = 27, p = 0.465; Table 8.1).

Furthermore, there was a significant positive correlation for $\delta^{13}C$ between host and parasite (Pearson correlation, r = 0.63, p < 0.001; Fig. 8.3A), but this relationship did not exist for $\delta^{15}N$ (r = -0.13, p = 0.509; Fig. 8.3B). Consequently, parasite enrichment scaled negatively with the $\delta^{15}N$ of the mussel (Pearson correlation, r = -0.75, p < 0.001; Fig. 8.4), while this relationship was not significant for $\delta^{13}C$ enrichment (r = -0.29, p = 0.130). Finally, there was no relationship between the enrichment of the parasite ($\Delta^{15}N$: parasite $\delta^{15}N$ – mussel $\delta^{15}N$) and the C:N ratio of the mussel (Pearson correlation, r = 0.06, p = 0.743).

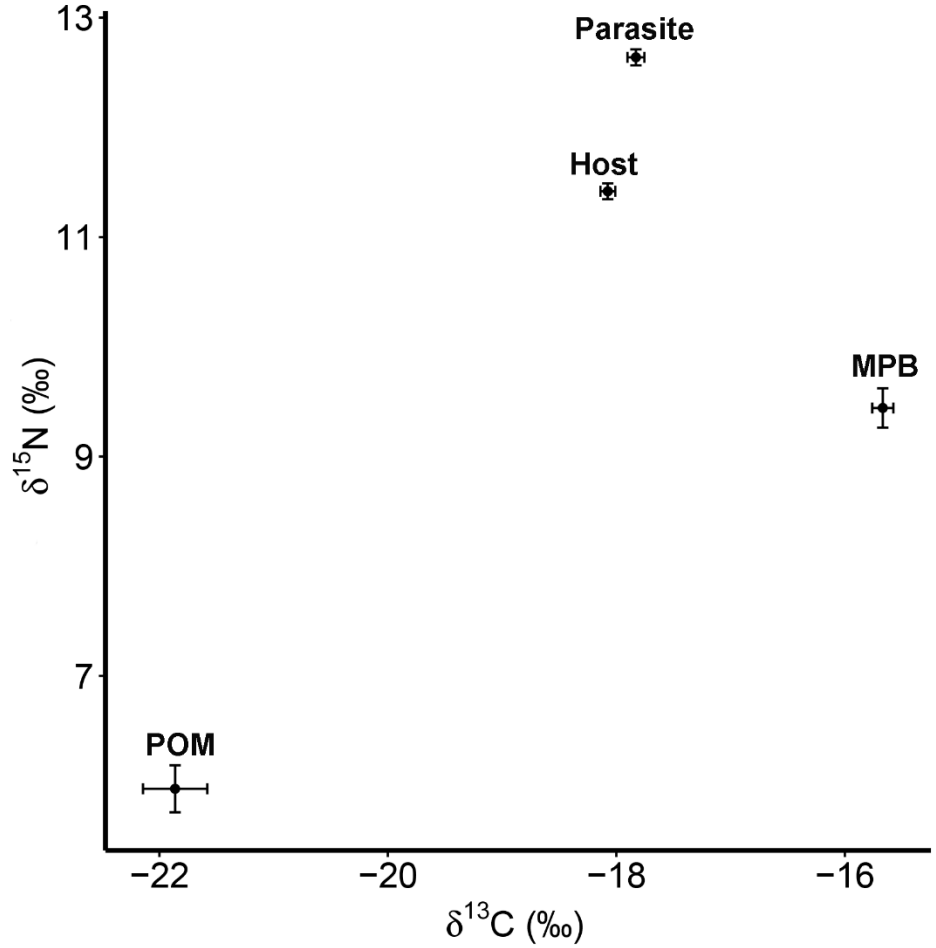

Fig. 8.2 Isotope values of $\delta^{15}N$ and $\delta^{13}C$ with standard errors for suspended particulate organic matter (POM), microphytobenthos (MPB), the blue mussel host (*Mytilus edulis*) and its intestinal parasite *Mytilicola orientalis.*

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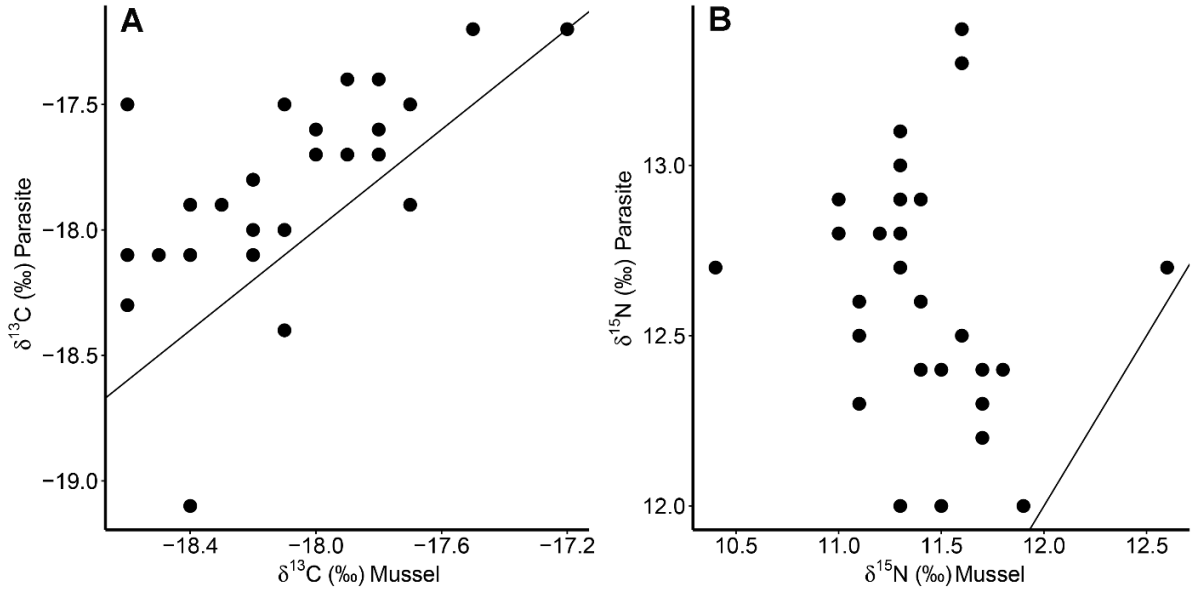

Fig. 8.3 Relationships of **A)** $\delta^{13}C$ and **B)** $\delta^{15}N$ for the native mussel host *Mytilus edulis* and the invasive parasite *Mytilicola orientalis.* Each dot represents values for one individual host-parasite pair. Black lines indicate the 1:1 relationship.

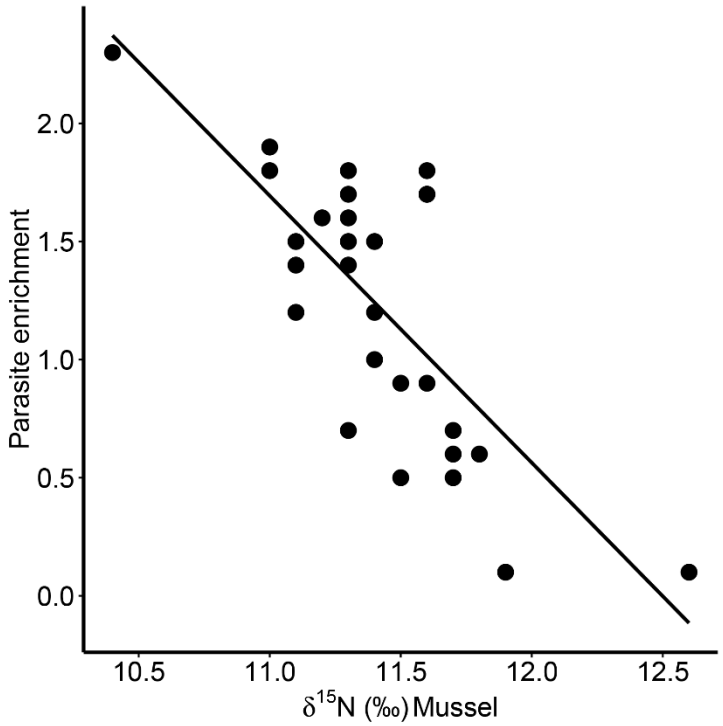

Fig. 8.4. Relationship between parasite enrichment ($\Delta^{15}N$ = parasite $\delta^{15}N$ – mussel $\delta^{15}N$) of the invasive parasite *Mytilicola orientalis* and the $\delta^{15}N$ of the blue mussel host *Mytilus edulis.* Each dot represents values for one individual host-parasite pair.

Mixing model

In the first mixing model, we used standard trophic fractionation factors of 3.4‰ for $\delta^{15}N$ and 1.0‰ for $\delta^{13}C$ for all diet sources (POM, MPB, mussel host). Results of this model showed slightly higher proportions for MPB (95% confidence interval; 27- 42%) than for mussel tissue (29-43%) and POM (27-33%) in the parasites' diet. However, when we adjusted the $\delta^{15}N$ fractionation factor for mussels, the relative contributions of all diet sources changed (Table 8.2; Fig. 8.5). By using a fractionation factor of 0-3‰ for $\delta^{15}N$ in the mixing model, tissue of the blue mussel host became the most important component in the parasites' diet with a probability that ranged from 54-96%. Yet, for a fractionation factor of 3.4 or 4‰ for $\delta^{15}N$, the model showed slightly higher proportions of MPB (range: 43-60%) in the diet of the parasite relative to blue mussel tissue (Table 8.2).

Table 8.2 Results of the sensitivity analysis of the mixing model. The trophic fractionation factor $\Delta^{15}N$ was in every model set at 3.4 ± 0‰ for POM (particulate organic matter) and MPB (microphytobenthos), while this factor was changed manually between 0.0 – 4.0‰ for the host (*Mytilus edulis*) tissue. The trophic fractionation factor $\Delta^{13}C$ was in all scenarios set at 1.0 ± 0‰. The table shows the diet proportions (95% confidence interval) of each of the three diet sources (host, MPB, POM), the hierarchical order of diet proportions in the parasites' (*Mytilicola orientalis*) diet and the probabilility of this ordering.

Trophic fractionation factor $\delta^{15}N$ (‰)	Host *Mytilus edulis* (%)	MPB (%)	POM (%)	Order	Probability (%)
0.0	61-89	1-20	8-21	Host > POM > MPB	96
1.0	58-84	3-22	12-22	Host > POM > MPB	91
2.0	43-66	13-32	19-27	Host > POM > MPB	54
3.0	32-48	24-39	25-32	Host > MPB> POM	64
3.4	29-43	27-42	27-33	MPB > Host > POM	43
4.0	25-36	31-45	28-35	MPB > POM > Host	60

Discussion

Our stable isotope analysis (SIA) showed that the intestinal parasitic copepod *Mytilicola orientalis* is enriched in $\delta^{15}N$ and $\delta^{13}C$ relative to its blue mussel (*Mytilus edulis*) host. While the enrichment in $\delta^{13}C$ was similar to the standard $\Delta^{13}C$ discrimination factor of about 1‰, the observed trophic enrichment ($\Delta^{15}N$) of 1.2‰ was considerably lower than the standard trophic fractionation factor of 3.4‰. As this standard value has been set to distinguish between trophic levels (e.g., Minagawa and Wada, 1984; Vander Zanden et al., 1997; Post et al., 2002), our results indicate that this intestinal parasite might not only feed on host tissue, but also on host gut content, suggesting a complex mix of a parasitic and commensal relationship in this new parasite-host association.

These findings differ from the results of a stable isotope analysis of a congeneric of *M. orientalis*, the copepod *M. intestinalis*, which also lives in the intestine of *M. edulis*. Gresty and Quarmby (1991) found $\delta^{15}N$ values of the parasite that were, on average, 2.8‰ higher than for the blue mussel and suggested a parasitic trophic relationship between the parasite and its host. In their study, infected mussels (collection season unknown) were kept in aquaria that were filled with estuarine water and mussels were fed with the diatom *Phaeodactilym trycornutum* 2-3 weeks prior to dissection, after which the mussel intestine was used in the SIA analysis. Therefore, variation in collection season, habitat, food source and analysed tissue could have led to differences in trophic fractionation factor between both parasite species. Nevertheless, the

feeding behaviour of both congeneric copepods might also be different, but the exact mechanism of the consumption of host tissue is for both parasite species not well known. It may be that *M. intestinalis* does not directly feed on host tissue but rather on sloughed-off cells of the intestine or on mucus produced by the host so that the parasite only indirectly feeds on the host (Gresty and Quarmby 1991). In contrast to *M. intestinalis*, the much lower trophic enrichment ($\Delta^{15}N$) of 1.2‰ observed in our study might suggest a more complex mix of a parasitic and commensal relationship between *M. orientalis* and its new host. This was also indicated by the results of the isotope mixing modelling, a statistical method that is increasingly used by ecologists (reviewed by Phillips et al., 2014). Generally, the results of our mixing models indicated that, in addition to host tissue (*M. edulis*; probabilities of 25 - 89%), suspended particulate organic matter (POM; 8 - 35%) and microphytobenthos (MPB; 1 - 45%) could make up substantial proportions of the parasites' diet. Both POM and MPB are important food sources for blue mussels (Dubois et al., 2007).

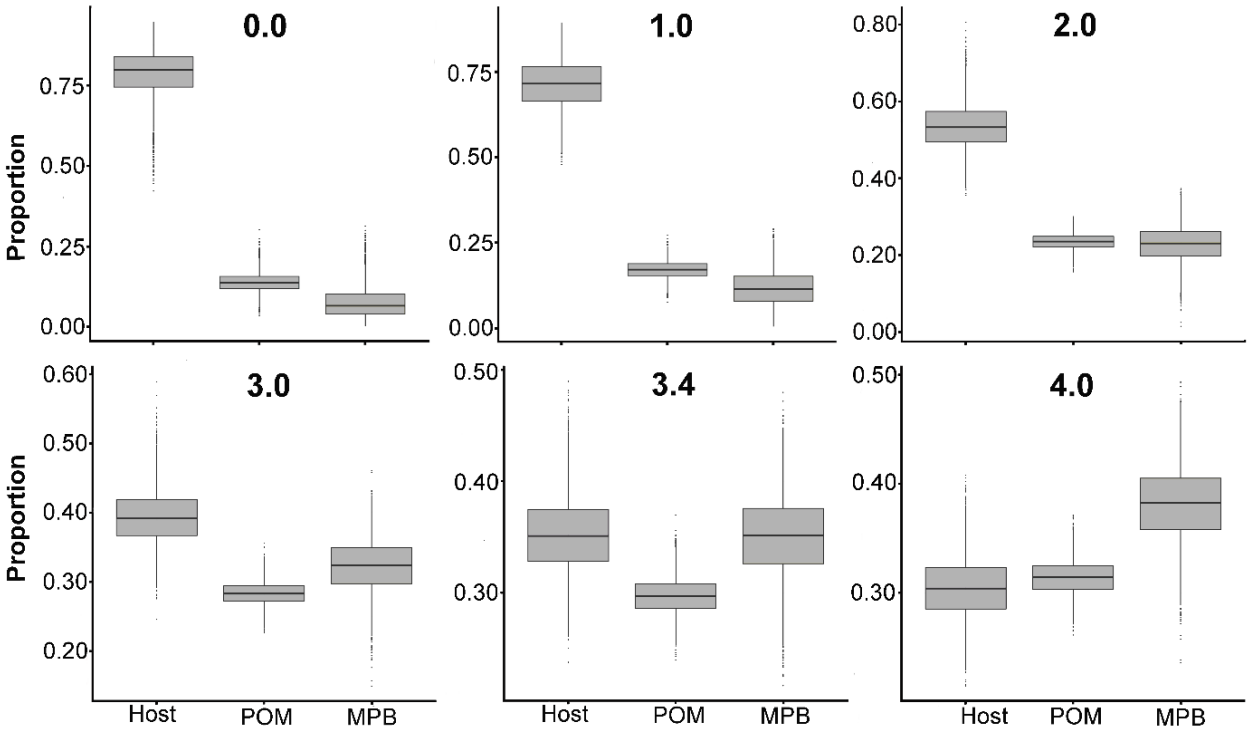

Fig. 8.5 Comparison of proportions of diet sources in the intestinal parasite *Mytilicola orientalis* by using different mixing models with a varying fractionation factor $\delta^{15}N$ (0.0, 1.0, 2.0, 3.0, 3.4 and 4.0‰) for the blue mussel *Mytilus edulis* host. In every model the trophic fractionation factor $\delta^{15}N$ for MPB and POM were set at 3.4‰, and the trophic fractionation factor of $\delta^{13}C$ was set at 1.0‰.

In the present study, mussel diet sources were sampled at other sites (Marsdiep and Balgzand) than mussels and parasites (Mok). However, during flood the three areas are tightly connected, when water from the North Sea is feeding the intertidal areas of Balgzand and Mok via the same deep channel (Marsdiep; Postma, 1954; Duran-Matute et al., 2014). Therefore, we assume that POM originating from this channel is incorporated in the mussel and parasite tissue 2-3 months later (Dubois et al., 2007; Phillips et al., 2014). MPB samples were collected at the same time as the POM samples, but on a sampling site located on the tidal flats of Balgzand, on

the opposite side of the channel feeding the Mok, where hosts with parasites were sampled. For the mixing models, we considered the samples from to Balgzand to be representative for the MPB available to the mussels. However, MPB is known to occur in higher abundances in the Mok than at Balgzand (4 g C m^{-2}; Borsje, 2006) and both areas are under the influence of different fresh water sources, however the exact impact of these discharges on the mussels' diet is yet unknown. Potential differences in the isotopic composition of MPB between the areas may introduce bias in our analyses but given the relatively small range of isotope signals observed in MPB in another study in the Wadden Sea (M. J. A. Christianen, pers. comm.), we consider the potential bias to be minor. Besides spatial differences, seasonality may be another potential factor known to affect isotope signals over a wide range of trophic levels (Kang et al., 2006; Cabanellas-Reboredo et al., 2009; Ezgeta-Balić et al., 2014; de la Vega et al., 2016), and like with fresh water discharge, is expected to affect $\delta^{15}N$ and $\delta^{13}C$ ratios of mussel diet sources, potentially confounding our mixing models. However, as we used time spans known to reflect typical tissue incorporation times, seasonal affects should not have compromised the results of our mixing models and our general conclusion of a mixed feeding mode of the parasites. Over the course of a year, the relative contributions of POM, MPB and mussel to the parasites' diet may of course change with season and/or salinity and to what extend this actually happens should be a topic of future studies.

For mixing models, the use of appropriate discrimination factors is essential (Phillips et al., 2014), however the trophic fractionation factor is known to scale negatively with trophic level (Caut et al., 2009; Hussey et al., 2014). The results of the mixing model sensitivity analyses, with a variety of trophic fractionation factors (1 - 4‰) for mussels, were relatively similar for fractionation factors between 1-3‰, with host tissue being the dominant diet source, while factors higher than 3‰ (i.e., 3.4 and 4‰) showed a dominance of MPB in the parasites' diet. Therefore, controlled lab experiments are needed to identify the exact diet sources and corresponding fractionation factors for the parasite *M. orientalis*, before accurate and reliable predictions of the diet proportions can be made with the use of mixing modelling (Phillips et al., 2014). That the actual trophic fractionation factor for *M. orientalis* may differ from the standard $\Delta^{15}N$ depending on the resource $\delta^{15}N$ is also evident from the observed negative scaling of the trophic enrichment in *M. orientalis* with host $\delta^{15}N$. Such a negative scaling relationship has also been observed within individual predators and their prey (Caut et al., 2009; Dennis et al., 2010) besides the general negative scaling relationship among predator-prey pairs detected in comparative studies (Caut et al., 2009; Hussey et al., 2014). However, the underlying mechanisms of both scaling relationships are not well understood (Caut et al., 2009; Hussey et al., 2014). In the case of *M. orientalis*, the issue is further complicated by the fact that $\delta^{13}C$ values of the parasite correlated positively, as expected for a trophic relationship, with those of their hosts, but that, surprisingly, this relationship did not exist for $\delta^{15}N$. Here, the variation in $\delta^{15}N$ values among individual *M. orientalis* samples was larger than the variation among individual mussels, resulting inevitably in a negative scaling relationship between parasite trophic enrichment ($\Delta^{15}N$) and $\delta^{15}N$ values of hosts. This suggests that *M. orientalis* might be relatively decoupled from its host nitrogen sources. However, why this is the case we can only speculate. Ratios of stable isotopes may change between parasite and host due to differential digestion or fractionation during assimilation and metabolic processes. For example, the parasite could selectively use alternative or depleted nitrogen compounds stored within the mussels (Barret, 1981), or bacteria in the gut of the mussel could cause substantial changes in the nitrogen cycle within the host or specialized nitrogen turnover processes within the parasite could cause potential decoupling between host and parasite. Future experiments will be needed to explore the exact mechanisms behind the nitrogen relationship in the new *M. orientalis*-mussel association.

In conclusion, our study indicates that the invasive parasite *M. orientalis* feeds on tissue of its new mussel host, but almost certainly also on the gut content of mussels (particulate organic matter and microphytobenthos), given the low trophic enrichment of 1.2‰. This conclusion was also supported by isotope mixing modelling results. However, those have been shown to be sensitive to the choice of trophic fractionation factors and the actual contribution of host tissue to the diet of *M. orientalis* differed depending on the fractionation factor used. Despite these caveats, stable isotope analysis combined with additional mixing models promises to provide a useful tool to identify the trophic relationships of new parasite-host associations that result from the increasing co-introductions of parasites with their hosts into new ecosystems.

Acknowledgments

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