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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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Summary

Invasive parasites can spill over to new hosts in invaded ecosystems with often unpredictable trophic relationships in the newly arising parasite-host interactions. In European seas, the intestinal copepod *Mytilicola orientalis* was co-introduced with Pacific oysters (*Magallana gigas*) and spilled over to native blue mussels (*Mytilus edulis*), with negative impacts on the condition of infected 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 mussel host tissue and the primary food sources of the mussels, microphytobenthos (MPB) and particulate organic matter (POM). The copepods were slightly enriched in $\delta^{15}N$ (mean $\Delta^{15}N \pm SD$; $1.22 \pm 0.58‰$) and $\delta^{13}C$ ($\Delta\ \delta^{13}C$ $0.25 \pm 0.32‰$) with respect to their host. Stable isotope mixing models using a range of trophic fractionation factors indicated that host tissue was the main food resource with consistent additional contributions of MPB and POM. These results suggest that the trophic relationship of the invasive copepod with its mussel host is parasitic as well as commensalistic. Stable isotope studies such as this one may be a useful tool to unravel trophic relationships in new parasite-host associations in the course of invasions.

Key words

parasite invasion; parasite co-introduction; parasite spillover; parasite-host interaction; Stable Isotope Analysis (SIA); trophic fractionation factor; mixing model; Pacific oyster; *Magallana gigas*

41 **Key findings**

42 - New parasite-host relationships can arise by parasite invasions
43 - The invasive parasite *Mytilicola orientalis* spilled over to native blue mussels
44 - Stable isotope analysis revealed an enrichment in $\delta^{15}N$ and $\delta^{13}C$ of the parasite compared
45 to the host
46 - Mixing models indicated hosts to be main resource with additional contributions of algae
47 consumed by hosts
48 - *M. orientalis* has a parasitic as well as a commensalistic relationship with blue mussels

49

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), 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) and the duration of the interaction (very short in the case of prey-predator systems and much longer in the case of parasite-host relationships; Dubois et al., 2009). 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's stomach contents or specific pre-digested biochemical compounds (Iken et al., 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 (partially) 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; Dubois et al., 2009) 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 ($\Delta\delta^{13}C$) is used to

73 determine the diet source of carbon (e.g., terrestrial vs marine primary producers; Hobson, 1986)
74 with a standard discrimination factor of 1.0‰, while trophic enrichment ($\Delta\delta^{15}N$) is used to
75 estimate the trophic position (Vander Zanden et al., 1997), in which a fixed value (also known as
76 the trophic fractionation factor) of 3.4‰ is most commonly used to analyse relative species
77 trophic levels (Minagawa and Wada, 1984; Vander Zanden et al., 1997; Post, 2002). However,
78 studies which compare isotopic signatures of parasites with their hosts indicate that parasites do
79 not always fit with the commonly accepted consumer-diet discrimination patterns seen in free-
80 living species (e.g. Iken et al., 2001; Power and Klein, 2004; Neilson and Brown, 1999; O'Grady
81 and Dearing, 2006; Xu et al., 2007; Dubois et al., 2009; Navarro et al., 2014, Behrmann-Godel
82 and Yohannes, 2015).

83 In this study, we analyse the trophic relationship between the invasive endoparasite *Mytilicola*
84 *orientalis* and its new host in European seas, the native blue mussel *Mytilus edulis* in the Dutch
85 Wadden Sea. This parasitic copepod has been recently co-introduced with aquaculture imports of
86 the invasive Pacific oyster (*Magallana* (previously *Crassostrea*) *gigas*) (Elsner et al., 2011) and
87 is known to spill over to native bivalves such as blue mussels and to a lesser extent to common
88 cockles (*Cerastoderma edulis*) and Baltic tellins (*Limecola* (formerly *Macoma*) *balthica*;
89 Goedknegt et al., 2017). *Mytilicola orientalis* was first described in the Sea of Japan (Mori,
90 1935) and has a direct life cycle with a short non-feeding free-living stage, after which it lives in
91 the intestines of its host. Here, the parasite is either feeding directly on the host tissue or
92 indirectly on host gut content, resulting in a reduction in body condition of infected blue mussels
93 (M. A. Goedknegt, unpublished results). As the exact diet source of the parasite is yet unknown,
94 we performed a SIA to clarify the trophic relationship between the parasite *M. orientalis* and its
95 new blue mussel host. Field samples of mussel hosts and parasites were analysed as well as the

96 two principal food sources of mussels, being particulate organic matter and microphytobenthos
97 (Dubois et al., 2007). This approach allowed us to determine the relative contributions of host
98 tissue and host food to the diet of the invasive copepod and to identify the trophic relationship of
99 this new parasite-host association that has resulted from the recent co-introduction of the
100 copepods with their oyster hosts.

101

102 **Material and methods**

103 *Collection of samples*

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

116 Microphytobenthos (MPB; n = 4 samples within an area of 50 m^2; Fig. 1) was sampled in the
117 beginning of July 2013 at an intertidal area south of the Marsdiep (Balgzand, Wadden Sea, The

118 Netherlands, Fig. 1) by collecting sediment from diatom mats into plastic bottles that were put on
119 ice and brought to the research facility. Extraction of microphytobenthic diatoms in the
120 laboratory was done by following the method of Riera and Richard (1996), slightly modified by
121 Herlory et al. (2007). The sediment was spread in a tray, covered by three layers of nylon mesh
122 (2 x 100 µm, 1 x 50µm) that was kept moist by repeatedly spraying filtered seawater on top. The
123 samples were then left in a temperature-regulated room overnight at 20ºC. The next morning, the
124 algae were washed into a beaker with filtered seawater. This solution was centrifuged (10 min at
125 10^3 G) and the remaining pellet was collected and stored at -20 °C.

126 Blue mussel and parasite (*Mytilicola orientalis*) samples were collected about three months later
127 than the POM and MPB samples (26 September 2013), to cover the minimum time it takes for
128 the diet to be incorporated into consumer tissue (Dubois et al., 2007; Phillips et al., 2014).
129 Mussels (n = 150) were collected from a mixed oyster and mussel bed located in the Mok (Fig.
130 1) and checked for presence of *M. orientalis* parasites under a magnification glass (magnification
131 3 - 8×). Mussels infected with at least two female parasites (n = 28 mussels), which can be more
132 than twice as large as males (Mori, 1935), were selected for the analysis, as a minimum of 0.4
133 mg dry weight of each pooled parasite and corresponding mussel sample (the adductor muscle of
134 the mussel) were required for the SIA. In these selected mussels, the mean *M. orientalis* intensity
135 (± SD) was 3.6 ± 1.8 and ranged between 2-9 copepods, with an average (± SD) ratio of 0.78 ±
136 0.21 females per infected mussel. Both parasite and matched mussel samples (each n = 28) were
137 then stored at -20 °C.

138

139 *Stable Isotope Analysis (SIA)*

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

149 The standard reference materials acetanilide (SD: $\delta^{15}N$ 0.3‰, $\delta^{13}C$ 0.1‰) and urea ($\delta^{15}N$ 0.2‰,
150 $\delta^{13}C$ 0.1‰) were respectively used as a correction and control of the isotope ratios found in the
151 samples. Isotope ratios of $\delta^{15}N$ and $\delta^{13}C$ were then expressed as permille (‰) differences from a
152 standard reference material using the formula $X = ((R_{sample}/R_{standard}) - 1) * 1000$, with R being the
153 ratio between the heavy and light isotopes of nitrogen (^{15}N:^{14}N) and carbon (^{13}C:^{12}C). The
154 reference material used for ^{15}N was atmospheric nitrogen N_2 and for ^{13}C Vienna Peedee-
155 Belemnite Limestone (vPDB).

156

157 *Statistical analysis*

158 Normality and homoscedasticity of the data were checked with histograms, qqplots and boxplots
159 (Zuur et al., 2010). Subsequently, differences in isotope ratios ($\delta^{13}C$ and $\delta^{15}N$) among the trophic
160 groups (POM, MPB, hosts, parasites) were analysed with ANOVA's and post-hoc Tukey tests.
161 Furthermore, comparisons and relationships between stable isotope data of parasites and

162 corresponding hosts ($\Delta\delta^{13}C$ and $\Delta\delta^{15}N$) and parasite intensity within the host were made using
163 paired Student's t-tests and Pearson correlations, respectively. All statistical analyses were
164 performed in the statistical software environment R (R Development Core Team, 2015).

165

166 *Isotope mixing models*

167 The relative contribution of diet sources in the consumers' diet can be determined by the use of
168 stable isotope mixing models (i.e., Phillips and Gregg, 2003; Inger et al., 2006). In this study, we
169 used an isotope mixing model to determine the relative contributions of host tissue (blue mussel)
170 and host gut content (represented by POM and MPB) to the diet of the parasitic copepod *M.*
171 *orientalis*. The package *simmr* (Parnell, 2016) was used to solve mixing equations for stable
172 isotopic data within a Bayesian framework in R (R Development Core Team, 2015). This
173 package allows the use of multiple diet sources with adjustable source specific trophic
174 fractionation factors. In the mixing model, individual $\delta^{15}N$ and $\delta^{13}C$ values of the parasite
175 samples were used as the consumer data. Diet source data included the mean (± SD) $\delta^{15}N$ and
176 $\delta^{13}C$ values of the sources POM, MPB and blue mussel, and were corrected for trophic
177 fractionation. This correction for trophic fractionation was done in two different ways: first, we
178 used the standard trophic fractionation factors of 3.4‰ for $\delta^{15}N$ and 1.0‰ for $\delta^{13}C$ for all diet
179 sources (Minagawa and Wada, 1984; Vander Zanden et al., 1997; Post, 2002), as controlled diet
180 studies and thus taxon-specific fractionation factors are not (yet) available for the parasite.
181 Second, we varied the trophic fractionation values used for $\delta^{15}N$ between 1 and 4‰ to determine
182 how much the estimated relative contribution of all diet sources changed with the fractionation
183 factor. This second approach served as a sensitivity analysis to account for the unknown 'real'

184 trophic fractionation factor of the parasites (see discussion for more details). Finally, we ran a
185 third mixing model approach where we used mussel, POM and MPB data from four seasons
186 from the long-term monitoring at our sampling site to identify whether seasonal changes of
187 mussel, POM and MPB isotope signals would change our results.

188

189 **Results**

190 *All trophic groups*

191 The four trophic groups (POM, MPB, mussel, parasite) differed significantly in $\delta^{15}N$ (ANOVA;
192 $F_{3,73} = 588.16$, $p < 0.001$) and $\delta^{13}C$ ($F_{3,73} = 200.41$, $p < 0.001$). Values of $\delta^{15}N$ were highest for
193 the parasitic copepod and lowest for POM, while for $\delta^{13}C$ MPB and POM had the highest and
194 lowest values, respectively (Table 1; Fig. 2).

195

196 *Parasites and hosts*

197 Parasitic copepods were significantly enriched in $\delta^{15}N$ and $\delta^{13}C$ with respect to their host, the
198 blue mussel (Student's paired t-test; $\delta^{15}N$: $t = 11.178$, $df = 27$, $p < 0.001$; $\delta^{13}C$: $t = 4.071$, $df = 27$,
199 $p < 0.001$; for means see Table 1). However, the levels of enrichment were relatively small
200 (mean ± SD; 1.22 ± 0.58‰ for $\delta^{15}N$ and 0.25 ± 0.32‰ for $\delta^{13}C$; Fig. 2). This minor enrichment
201 of the parasite in relation to its host was not reflected in the differences in mean total nitrogen
202 (TN) and total organic carbon content (TOC) in both tissues (Student's t-test; TN (%): $t = -1.361$,
203 $df = 27$, $p = 0.185$; TOC (%): $t = -0.741$, $df = 27$, $p = 0.465$; for means see Table 1).

204

205 Furthermore, there was a significant positive correlation for $\delta^{13}C$ between host and parasite
206 (Pearson correlation, $r = 0.63$, $p < 0.001$; Fig. 3A), but this relationship did not exist for $\delta^{15}N$ (r =

207 -0.13, $p = 0.509$; Fig. 3B). Consequently, parasite enrichment ($\Delta\delta^{15}N$: parasite $\delta^{15}N$ – mussel

208 $\delta^{15}N$) scaled negatively with mussel $\delta^{15}N$ mussel (Pearson correlation, $r = -0.75$, $p < 0.001$; Fig.

209 4), while this relationship was not significant for $\delta^{13}C$ enrichment ($r = -0.29$, $p = 0.130$). In

210 addition, there was no relationship between the enrichment of the parasite ($\Delta^{15}N$: parasite $\delta^{15}N$ –

211 mussel $\delta^{15}N$) and the C:N ratio of the mussel (Pearson correlation, $r = 0.06$, $p = 0.743$). Finally,

212 in our dataset parasite intensity in infected hosts did not affect the $\delta^{15}N$ ($r = 0.35$, $p = 0.064$) nor

213 $\delta^{13}C$ ($r = 0.07$, $p = 0.708$) signals of the parasites and neither those of the hosts ($\delta^{15}N$: $r = -0.07$, p

214 = 0.719, $\delta^{13}C$: $r = -0.19$, $p = 0.334$).

215

216 *Isotope mixing models*

217 In the first mixing model, we used standard trophic fractionation factors of 3.4‰ for $\delta^{15}N$ and

218 1.0‰ for $\delta^{13}C$ for all diet sources (POM, MPB, mussel host). Results of this model showed that

219 mussel tissue was the main contributor to the parasites' diet (95% confidence interval; 45-52%),

220 with lower contributions by POM (30-35%) and MPB (15-25%). When we varied the $\delta^{15}N$

221 fractionation factors in the second run of the mixing models, the relative contributions of all diet

222 sources changed (Table 2; Fig. 5) but for fractionation factors between 0 and 3.4‰ for $\delta^{15}N$ this

223 did not affect the dominance of mussel host tissue in the parasites' diet. Only for a fractionation

224 factor of 4‰ for $\delta^{15}N$, the model showed higher proportions of POM (37-42%) and MPB (26-

225 37%) in the diet of the parasite relative to blue mussel tissue (24-35%; Table 2; Fig. 5). Finally,

226 using isotope values of mussels, POM and MPB from four seasons/months at our sampling site

227 (March, June, September and December 2014) in the main mixing model (3.4‰ for $\delta^{15}N$ and

228 1.0‰ for $\delta^{13}C$) did not change the results qualitatively (Table S1).

229

230 **Discussion**

231 Our stable isotope analysis (SIA) showed that the intestinal parasitic copepod *Mytilicola*
232 *orientalis* is enriched in $\delta^{15}N$ and $\delta^{13}C$ with respect to its blue mussel (*Mytilus edulis*) host. Yet,
233 for both isotopes, the observed enrichment of the parasite compared to its host (1.2‰ for $\Delta\delta^{15}N$
234 and 0.25‰ for $\Delta\delta^{13}C$) was considerably lower than the standard trophic fractionation factor of
235 3.4‰ for $\Delta\delta^{15}N$ and the standard discrimination factor of about 1‰ for $\Delta\delta^{13}C$, which are
236 commonly used to distinguish between trophic levels (e.g. Minagawa and Wada, 1984; Vander
237 Zanden et al., 1997; Post, 2002). Given that these values are also appropriate for the parasites,
238 this would indicate that this intestinal parasite does not only feed on host tissue, but also on host
239 gut content, suggesting a complex mix of a parasitic and commensal relationship in this new
240 parasite-host association. Such a mixed diet was also indicated by the results of the stable isotope
241 mixing modelling, a statistical method that is increasingly used by ecologists (reviewed by
242 Phillips et al., 2014). Generally, the results of our mixing models (using standard fractionation
243 factors) demonstrated that host tissue dominated with suspended particulate organic matter and
244 microphytobenthos contributing to the parasites' diet. However, alternatively, the relatively
245 small signals of enrichment may not result from mixed diet contributions but could also suggest
246 parasite specific lower trophic fractionation patterns. Indeed, smaller than standard enrichment
247 patterns have previously been found in other parasite-host systems (O'Grady and Dearing, 2006;
248 Dubois et al., 2009; Yurlova et al., 2014; Behrmann-Godel and Yohannes, 2015; Demopoulos
249 and Sikkel, 2015), including parasites with a strict parasitic way of life such as trematodes
250 (Dubois et al., 2009). To investigate the effect of potentially lower and higher than usual trophic
251 fractionation factors, we conducted a second run of mixing models using a variation of trophic
252 fractionation values (0-4‰). These models showed that mussels were still the dominant food

253 source in all but the highest fractionation value (4‰). In addition, the positive correlation in
254 carbon signatures between parasite and host suggests that the host represents a major carbon
255 source for the parasitic copepod. All these results confirm that the parasite has, at least to a large
256 extent, a parasitic trophic relationship with its host. This would also explain the negative effect
257 of the parasite on host body condition which has been previously observed in controlled
258 laboratory experiments (M. A. Goedknegt, unpublished results). However, in all the scenarios of
259 the mixing models developed in our study, host tissue (*M. edulis*; proportions of 24-99%) was
260 never the only resource of *M. orientalis* but host gut content, represented by suspended
261 particulate organic matter (POM; 0 - 42%) and microphytobenthos (MPB: 0-37%), consistently
262 contributed to the parasite's diet. This suggests that the trophic relationship of the parasite with
263 its new host is also partly commensalistic. The exact contributions of the diet under different
264 environmental conditions as well as the resulting diet specific trophic fractionation factors
265 remain to be experimentally studied (although this will be logistically challenging, see below).
266
267 Our findings differ from the results of a stable isotope analysis of a congeneric species of *M.*
268 *orientalis*, the copepod *M. intestinalis*, which also lives in the intestine of *M. edulis*. Gresty and
269 Quarmby (1991) found $\delta^{15}N$ values of the parasite that were, on average, 2.8‰ higher than for
270 the blue mussel and suggested a parasitic trophic relationship between the parasite and its host.
271 In their study, infected mussels (collection season unknown) were kept in aquaria that were filled
272 with estuarine water and mussels were fed with the diatom *Phaeodactilym trycornutum* 2-3
273 weeks prior to dissection, after which the mussel intestine was used in the SIA analysis.
274 Methodological differences may underlie the diverging trophic fractionation factors in the two
275 parasite species but it is also possible that the feeding behaviour of both congeneric copepods is

276 different. Although *M. intestinalis* may not directly feed on host tissue but rather on sloughed-off
277 cells of the intestine or on mucus produced by the host (Gresty and Quarmby, 1991), it may still
278 mainly feed (indirectly) on its mussel hosts. In contrast, the much lower trophic enrichment
279 ($\Delta\delta^{15}N$) of 1.2‰ observed in *M. orientalis* in our study might suggest a more complex mix of a
280 parasitic and commensal relationship between this parasite and its new host. A direct comparison
281 of the two parasites in future experimental stable isotope studies would be interesting and could
282 help to identify potential differences in diet composition of the two related parasite species.
283

284 In the present study, mussel diet sources were sampled at other sites (Marsdiep and Balgzand)
285 than mussels and parasites (Mok). However, during flood the three areas are tightly connected,
286 when water from the North Sea is feeding the intertidal areas of Balgzand and Mok via the same
287 deep channel, the Marsdiep (Postma, 1954; Duran-Matute et al., 2014). Therefore, we assume
288 that POM originating from this channel is incorporated in the mussel and parasite tissue 2-3
289 months later (Dubois et al., 2007; Phillips et al., 2014). MPB samples were collected at the same
290 time as the POM samples, but on a sampling site from a seasonal isotope monitoring study
291 located on the tidal flats of Balgzand, on the opposite side of the channel feeding the Mok, where
292 hosts with parasites were sampled. For the stable isotope mixing models, we considered the
293 samples from Balgzand to be representative for the MPB available to the mussels. However,
294 MPB is known to occur in higher abundances in the Mok than at Balgzand (4 g C m^{-2}; Borsje,
295 2006) and both areas are under the influence of different fresh water sources. The exact impact
296 of these discharges on the mussels' diet is yet unknown. Potential differences in the isotopic
297 composition of MPB between the areas may introduce bias in our analyses, but given the
298 relatively small range of isotope signals observed in MPB on local scales as observed in a recent

299 large-scale isotope study along the entire Dutch Wadden Sea (Christianen et al., 2017; M. J. A.
300 Christianen, pers. communications), we are confident that the spatial mismatch in sampling
301 location is not adding a severe bias in MPB measurements. Besides spatial differences,
302 seasonality may be another potential factor known to affect isotope signals over a wide range of
303 trophic levels (Kang et al., 2006; Cabanellas-Reboredo et al., 2009; Ezgeta-Balić et al., 2014; de
304 la Vega et al., 2016), and expected to affect $\delta^{15}N$ and $\delta^{13}C$ ratios of mussel diet sources,
305 potentially confounding our mixing models. However, data from a seasonal isotope investigation
306 in our study area from which the POM/MPB data originated, suggest a limited effect of
307 seasonality on our results. Preliminary results of this seasonal study showed only small
308 differences in isotope values between June and September for POM ($\Delta\delta^{15}N = 0.01$; $\Delta\delta^{13}C = 0.9$)
309 and some larger differences for MPB ($\Delta\delta^{15}N = 1.7$; $\Delta\delta^{13}C = 1.6$; A. S. Jung, pers.
310 communications). These results demonstrate that the growing season of various phytoplankton
311 species did not lead to strong changes in the isotopic signals of POM during the summer. For
312 MPB on the other hand, the changes are larger and here a switch in microphytobenthos species
313 composition may have caused a change of isotopic values during the summer. However, the
314 seasonal change in isotopic values of these diet sources only affected the isotopic signal of the
315 host *Mytilus edulis* to a small extent between June and September ($\Delta\delta^{15}N = 0.1$; $\Delta\delta^{13}C = 0.9$; A.
316 S. Jung, pers. communications). As especially the $\delta^{15}N$ values of mussels barely changed during
317 the summer, we do not believe that seasonality effects are confounding our analyses, in particular
318 with respect to our main focus of investigation, the trophic relationship of the parasite *M.*
319 *orientalis* with its mussel host. Our inferences are further supported by stable isotope mixing
320 models in which we used original data for POM and MPB from the isotope study of four
321 different seasons to investigate how this would change the results. These analyses showed that

322 mussels remain the main food source for the parasites, independent of the season (see electronic
323 appendix Table S1). However, over the course of a year, the relative contributions of POM, MPB
324 and mussel to the parasites' diet may of course change with season and/or salinity and to what
325 extend this actually happens should be a topic of future studies.
326
327 For isotope mixing models, the use of appropriate discrimination factors is essential (Phillips et
328 al., 2014) but, as mentioned above, parasites may show enrichments patterns different from free-
329 living species (O'Grady and Dearing, 2006; Dubois et al., 2009; Yurlova et al., 2014;
330 Demopoulos and Sikkel, 2015) and we accounted for this using mixing models with different
331 fractionation factors (see above). However, further diversion from standard enrichment patterns
332 may arise from the universal pattern that trophic fractionation factors of consumers are known to
333 scale negatively with the isotope ratio of their resource (Caut et al., 2009; Hussey et al., 2014).
334 The same negative scaling was observed in our data with the trophic enrichment in *M. orientalis*
335 decreasing with host $\delta^{15}N$. Such a negative scaling relationship between resource $\delta^{15}N$ and
336 consumer trophic enrichment has also been observed within individual predators and their prey
337 (Caut et al., 2009; Dennis et al., 2010) besides the general negative scaling relationship among
338 species observed in comparative studies (Caut et al., 2009; Hussey et al., 2014). However, the
339 underlying mechanisms of both scaling relationships are not well understood (Caut et al., 2009;
340 Hussey et al., 2014). In the case of *M. orientalis*, the issue is further complicated by the fact that
341 $\delta^{13}C$ values of the parasite correlated positively, as expected for a trophic relationship, with those
342 of their hosts, but that, surprisingly, this relationship did not exist for $\delta^{15}N$. Here, the variation in
343 $\delta^{15}N$ values among individual *M. orientalis* samples was larger than the variation among
344 individual mussels, resulting inevitably in a negative scaling relationship between parasite

345 trophic enrichment ($\Delta\delta^{15}N$) and $\delta^{15}N$ values of hosts. This suggests that *M. orientalis* might be
346 relatively decoupled from its host nitrogen sources. However, why this is the case we can only
347 speculate. Ratios of stable isotopes may change between parasite and host due to differential
348 digestion or fractionation during assimilation and metabolic processes. For example, the parasite
349 could selectively use alternative or depleted nitrogen compounds stored within the mussels
350 (Barret, 1981), bacteria in the gut of the mussel could cause substantial changes in the nitrogen
351 cycle within the host or specialized nitrogen turnover processes within the parasite could cause
352 potential decoupling between host and parasite. Alternatively, our sample choice of selecting
353 mostly larger females (which was necessary to obtain sufficient parasite tissue for the SIA
354 analysis), could also have affected the relatively large variation in $\delta^{15}N$ we have observed in
355 parasite samples. Possibly, females exhibit different stable isotope composition than males due to
356 differences in body size (growth rates) and feeding rates, as well as due to egg production by
357 females. In addition, the natural variation in *M. orientalis* intensities in the selected hosts could
358 have influenced the variation in nitrogen among parasites, but this correlation was not significant
359 in our analyses. Controlled laboratory experiments may be needed to explore the exact
360 mechanisms behind the stable isotope patterns observed in the new *M. orientalis*-mussel
361 association. However, such experimental approaches with parasites are logistically challenging.
362 In particular in the case of the parasitic copepod in our study, it will be difficult to
363 experimentally disentangle the relative contributions of host mussel gut content (POM and MPB)
364 and host tissue to the parasite's diet, as the parasite inhabits the gut of the mussel host and has
365 access to both resources at the same time. Hence, if one feeds mussels with a different dietary
366 source, the parasite will have access to the mussel diet and at the same time feed on mussel
367 tissue. Thus, the parasite acquires isotopes that are a mix of both food sources. Also conducting

368 the usual diet switch studies with parasites is difficult, as the parasites cannot live without their
369 hosts. Hence, letting parasites feed on algae without a host is impossible and due to host
370 specificity changing hosts is also an issue. Such logistical complications due the natural history
371 of parasitic organisms are probably the reason why most isotope studies of parasites so far have
372 not used any experimental approaches. However, these studies still show that also samples from
373 the field can give some insight into how isotopes reflect trophic relationships of parasites and
374 their hosts. In our case, the combination of careful interpretation of the data and sensitivity
375 analyses using stable isotope mixing models allow for valid inferences in absence of
376 experimental data.

377

378 In conclusion, our study indicates that the invasive parasite *M. orientalis* mainly feeds on tissue
379 of its new mussel host, but, to a lesser extent, also on the gut content of mussels (represented by
380 particulate organic matter and microphytobenthos). This conclusion was also supported by stable
381 isotope mixing models which used various trophic fractionation values to account for potentially
382 different isotope enrichment patterns in parasitic compared to free-livings species. We propose
383 that stable isotope analysis combined with additional stable isotope mixing models promises to
384 provide a useful tool to explore the trophic relationships of new parasite-host associations that
385 result from the increasing co-introductions of parasites with their hosts into new ecosystems.

386

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398

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