

1 **Title:** Just hitching a ride: stable isotopes reveal non-feeding behaviour of *Anisakis simplex*  
2 within its host fish

3 **Running title:** *Anisakis* not feeding in host fish

4 **Authors' names and affiliations:**

5 Amandine Sabadel<sup>1,2,3,\*</sup>, Melanie Gay<sup>4</sup>, Henry S. Lane<sup>3</sup>, Odile Bourgau<sup>4</sup>, Sarah J. Bury<sup>3</sup>,  
6 Josette Delgado<sup>3</sup>, and Maureen Duflot<sup>4,5</sup>

7 <sup>1</sup>University of Otago, Dunedin, New Zealand

8 <sup>2</sup>Auckland University of Technology, Auckland, New Zealand

9 <sup>3</sup>National Institute for Water and Atmospheric Research, 301 Evans Bay Parade, Hataitai,  
10 Wellington 6021, New Zealand

11 <sup>4</sup>Anses, Laboratory for Food Safety, Boulogne-sur-Mer, France

12 <sup>5</sup>JRU Parasite Immunology and Molecular Biology (BIPAR), ENVA-INRAE-ANSES,  
13 Animal Health Laboratory, Maisons-Alfort, France

14 \*corresponding author; [amandine.sabadel@aut.ac.nz](mailto:amandine.sabadel@aut.ac.nz)

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24 **Data Availability Statement**

25 The data that support the findings of this study are openly available in figshare at

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27 **Conflict of interest statement**

28 None

29 **Abstract**

30 *Anisakis simplex* larvae, commonly found in marine fish, cause anisakiasis in humans,  
31 resulting in gastric to gastro-allergic symptoms. Despite known health risks, the impact of  
32 Anisakidae larvae on fish hosts is less understood. This study aimed to investigate this  
33 interaction by assessing the feeding strategy of *A. simplex*. *Anisakis* larvae were isolated from  
34 North Sea *Merluccius merluccius* tissues (stomach, body cavity, liver, muscle) and were  
35 analysed for carbon ( $\delta^{13}\text{C}$ ) and nitrogen ( $\delta^{15}\text{N}$ ) isotope values. Significant differences in  $\delta^{13}\text{C}$   
36 values were found among host tissues, with the liver differing from muscle and stomach  
37 tissues. In contrast, no differences were noted for the associated parasites. Additionally,  $\delta^{15}\text{N}$   
38 values indicated that the host occupied a significantly higher relative trophic position than its  
39 parasite. This suggests a lack of direct nutrient transfer from host to parasite, as the parasite  
40 would typically exhibit higher stable isotope values than the tissue they feed on. Therefore,  
41 *A. simplex*' stable isotope values might reflect those of its previous host (crustacean and/or  
42 small fish), providing insights into diet and movement of the paratenic *M. merluccius* host.  
43 Further research is needed to confirm these findings across different fish species and to  
44 explore *A. simplex* as a proxy for trophic ecology.

45 **Key words:** Anisakidae, fish host, hake, feeding behaviour, nutrient,  $\delta^{15}\text{N}$ ,  $\delta^{13}\text{C}$ , carbon,  
46 nitrogen, North Sea.

47 **Introduction**

48 Herring worm *Anisakis simplex* (Rudolphi, 1809), in its third larval stage, is a common  
49 nematode parasite found in a wide range of marine fish species (Davey, 1971; Gay et al.,  
50 2018; Mattiucci et al., 2018). Consumption of raw or undercooked seafood may lead to  
51 *A. simplex* infestation in consumers, known as anisakiasis. The ingestion of larvae may  
52 provoke gastric, intestinal or gastro-allergic symptoms in humans (EFSA, 2010; Levsen et al.,  
53 2018; Shamsi & Barton, 2023). Anisakiasis is a serious zoonotic disease with cosmopolitan  
54 distribution, and there has been a significant increase in its reported prevalence in the world  
55 over the last two decades (Bao et al., 2019; Caldeira, Pereira Alves, & Santos, 2021; Chai,  
56 Darwin Murrell, & Lymbery, 2005; Fiorenza et al., 2020).

57 While human health hazards associated with Anisakidae are well-known (Rahmati et al.,  
58 2020), relations between fish host and Anisakidae larvae remain less understood (Levsen et  
59 al., 2018), impacting not only organism health and behaviour (Zoratto et al., 2022) but also  
60 flesh quality and marketability (Bao et al., 2018). Fish acquire *A. simplex* larvae through  
61 feeding on infected crustacean (e.g. krill, copepods) or small fish (Dupouy-Camet, 2020).  
62 The larvae migrate from the stomach and end up in the organs of the body cavity (liver,  
63 gonad, mesenteries), and some of these larvae may migrate into the muscle (Cipriani et al.,  
64 2024; Dupouy-Camet, 2020). Anisakidae larvae have a rudimentary mouth comprising a  
65 boring tooth and an oesophagus (Kinkelin et al., 2014; Van Hien et al., 2021). However, it is  
66 unclear if they actively feed in a predator-prey manner, consuming or digesting tissues of  
67 their host fish for sustenance; or if they acquire nutrients by absorption, selecting only the  
68 molecules they need, akin to other parasites (Read, 2015).

69 Stable isotopes values of carbon ( $\delta^{13}\text{C}$ ) and nitrogen ( $\delta^{15}\text{N}$ ) have been extensively used in  
70 ecological studies (Boecklen et al., 2011) to unravel diet and animal movements (Hobson,  
71 2019; MacKenzie et al., 2011; Shipley & Matich, 2020). More recently, they have been

72 proposed as a powerful tool to study host-parasite interactions (Sabadel, Stumbo, &  
73 MacLeod, 2019), offering a means to trace the flow of nutrients by looking at predictable  
74 changes in  $\delta^{13}\text{C}$  and/or  $\delta^{15}\text{N}$  values between a parasite and its host, thus unravelling the  
75 dynamics of energy transfer within parasitic systems (Deudero, Pinnegar, & Polunin, 2002;  
76 Nachev et al., 2017; Porter et al., 2023; Sabadel et al., 2022; Sabadel & MacLeod, 2021). The  
77 method can also indicate the feeding mechanism of the parasite i.e. active versus absorptive  
78 feeding (Gilbert et al., 2020; Sabadel & MacLeod, 2021).

79 Using a stable isotope approach, this study aimed to 1) identify whether *A. simplex* feeds  
80 actively or through absorption; 2) qualify protein and lipid exchanges between *A. simplex* and  
81 its host fish; and 3) trace the movement of *A. simplex* through different host fish tissues.

## 82 **Materials and methods**

83 Whole *A. simplex* ( $n \leq 10$  larvae per organ) were isolated from the stomach, body cavity, liver,  
84 and muscle of freshly caught hake (*Merluccius merluccius*) ( $n=10$ ) from the North Sea in  
85 June 2023. Parasites which were clearly encapsulated into a specific tissue (stomach, liver,  
86 and muscle) were taken, along with an uninfected piece of the same host tissue directly  
87 adjacent to the infection site, to represent the expected food source of *A. simplex* (Paoletti et  
88 al., 2017; Pinnegar, Campbell, & Polunin, 2001). This is because different tissues in a fish  
89 have different stable isotope values and C/N ratios and different turnover times, so one tissue  
90 cannot serve as a proxy for the others (Mohan et al., 2016; Wyatt et al., 2019). However,  
91 parasites found in the body cavity (i.e., free-floating parasite), had no associated host tissue,  
92 therefore they were extracted alone. All larvae were immediately cleaned of their host tissue  
93 and rinsed in physiological saline. All samples were then dried in an oven at 50°C for 48 h  
94 and were later analysed individually for elemental composition (%C and %N) and stable  
95 isotope values ( $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ) at the NIWA Environmental and Ecological Stable Isotope  
96 Analytical Facility, Wellington, New Zealand, following routine analytical procedures

97 previously described (Bury et al., 2024; Stewart et al., 2023). Values of  $\delta^{13}\text{C}$  were not lipid-  
98 corrected to fully capture C-containing molecule exchanges (Sabadel et al., 2022). Moreover,  
99 between 7 and 10 Anisakidae larvae per fish were isolated from the same fish for molecular  
100 identification. qPCR, specific for *Anisakis simplex* s.s. was carried out according to (Paoletti  
101 et al., 2017).

## 102 **Results**

103 Forty-four parasites were isolated for molecular identification. All were identified as *A.*  
104 *simplex* s.s. A total of 38 samples, each containing multiple *A. simplex* specimens, were  
105 extracted from each fish part: stomach (n=10), liver (n=10), muscle (n=10) and body cavity  
106 (n=8) and were analysed for stable isotope values along with 30 matching fish tissues (n=10  
107 samples per tissue, except for body cavity). All mean  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values, C/N ratios, and  
108 the difference between parasite and host  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values are displayed **Error!**  
109 **Reference source not found.** and are visually represented in **Error! Reference source not**  
110 **found..** Significant differences were observed between host tissues in all variables:  $\delta^{13}\text{C}$   
111 values (One-way ANOVA,  $F_{2,27} = 110.665$ ,  $p < 0.001$ ),  $\delta^{15}\text{N}$  values (One-way ANOVA,  $F_{2,27}$   
112  $= 5.463$ ,  $p < 0.01$ ) and C/N ratios (One-way ANOVA,  $F_{2,27} = 41.810$ ,  $p < 0.001$ ), mostly  
113 driven by liver values (Tukey post hoc test). In contrast, no significant differences were found  
114 for  $\delta^{13}\text{C}$  (One-way ANOVA,  $F_{3,34} = 0.268$ ,  $p = 0.848$ ),  $\delta^{15}\text{N}$  values (One-way ANOVA,  $F_{3,34} =$   
115  $0.231$ ,  $p = 0.874$ ), or C:N ratio (One-way ANOVA,  $F_{3,34} = 0.668$ ,  $p = 0.578$ ) among *A. simplex*  
116 sampled from the different tissues, including the body cavity. All host tissue  $\delta^{15}\text{N}$  values  
117 were higher than parasite values. In contrast, host liver  $\delta^{13}\text{C}$  values were lower than parasite  
118 values, whilst muscle and stomach tissues had higher  $\delta^{13}\text{C}$  values compared to those of the  
119 parasites. Finally, the standard deviations associated with  $\delta^{15}\text{N}$  values in each host tissue were  
120 relatively small, ranging from 0.30 to 0.55‰. In contrast, the standard deviations of parasite  
121  $\delta^{15}\text{N}$  values had much greater variability, ranging from 0.83 to 2.45‰.

## 122 Discussion

123 Stable isotope values are affected by metabolic processes and trophic exchanges. Indeed,  
124 fractionation in predator-prey relationships is relatively predictable. In host-parasite  
125 interactions, however, fractionations vary significantly (Sabadel, Stumbo, & MacLeod, 2019;  
126 Thieltges et al., 2019), influenced by phylogeny (Kamiya, Urabe, & Okuda, 2020;  
127 Riekenberg et al., 2020), attachment site i.e., host guts versus host muscle (Riekenberg et al.,  
128 2020), and parasite feeding mode (Gilbert et al., 2020; Sabadel & MacLeod, 2021).

129 There was no significant difference in  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values between *A. simplex* samples  
130 collected from different host organs, contrasting with the variations observed in matching  
131 host tissues (Figure 1). This discrepancy suggested a lack of obvious transfer of C-containing  
132 or N-containing molecules directly from host organ tissues to the attached parasites. Notably,  
133 a variation in *A. simplex*  $\delta^{13}\text{C}$  values could be expected given the significant difference in  
134 host liver versus stomach and muscle  $\delta^{13}\text{C}$  values. Additionally, host  $\delta^{15}\text{N}$  values were  
135 significantly higher than those of the parasite, indicative of the host's higher relative trophic  
136 position. Absorptive parasites can selectively favour energy-efficient  $^{14}\text{N}$ -containing  
137 molecules, resulting in lower  $\delta^{15}\text{N}$  values in their tissues (Deudero, Pinnegar, & Polunin,  
138 2002; Nachev et al., 2017) or neutral discrimination against the host's values (Riekenberg et  
139 al., 2020). This process can also influence the stable isotope composition of host tissues. For  
140 instance, the presence of an absorptive-feeding parasite has been shown to enrich host tissue  
141  $\delta^{13}\text{C}$  values without affecting  $\delta^{15}\text{N}$  values in the case of a trematode (Sabadel & MacLeod,  
142 2021), whereas no significant stable isotope differences were observed with an  
143 acanthocephalan parasite (Molbert & Goutte, 2022). Another study on absorptive-feeding  
144 parasitic barnacles demonstrated the impact on adjacent tissues due to the parasite's presence  
145 and the host's immune response (Sabadel et al., 2022). Additionally, absorptive-feeding pike  
146 tapeworms were found to affect the overall host metabolism, leading to changes in tissue

147 stable isotope turnover rates (Yohannes et al., 2017). However, despite these possible  
148 interactions, typical parasite-host stable isotope offsets are only a few per mil units e.g. -1.0  
149 to 3.7‰ across various fish nematode species (Kamiya, Urabe, & Okuda, 2020). In our study,  
150 we observed a substantial discrepancy averaging  $5.6 \pm 0.3$ ‰ across all tissues (Table 1). This  
151 suggests that *A. simplex* likely obtained its low  $\delta^{15}\text{N}$  values from its previous host e.g.,  
152 crustacea or small fish, which are at a lower trophic level with lower  $\delta^{15}\text{N}$  values than *M.*  
153 *merluccius*. Altogether, these results suggest that *A. simplex* larvae are not taking up nutrients  
154 from their fish hosts, as previously suspected by Davey and Kan (1968).

155 Fish are considered paratenic hosts of *A. simplex*. Paratenic hosts offer a means of transport  
156 or a temporary refuge for the parasite, allowing it to move through the environment and  
157 facilitating the access to its definitive host, without any physiological changes (Anderson,  
158 2000). This “standby” state is likely to have low energy requirements. Our results support  
159 previous studies investigating the metabolism of *A. simplex* L3 larvae (Łopieńska-Biernat et  
160 al., 2019; Łopieńska-Biernat, Żółtowska, & Rokicki, 2006). *Anisakis simplex* L3 in paratenic  
161 hosts, which have a low body temperature of about 4°C, go into ‘hypometabolism’ and start  
162 synthesising trehalose using endogenous carbohydrate reserves stored from their previous  
163 hosts as a precursor (Łopieńska-Biernat et al., 2019; Łopieńska-Biernat, Żółtowska, &  
164 Rokicki, 2006). This would translate isotopically to no significant changes in the parasite’s  
165  $\delta^{13}\text{C}$  values if no waste was produced. Or, if waste was produced, to a potentially predictable  
166 fractionation (-2 to -3‰) as has been observed for glucose synthesis for other species of  
167 parasites (Hesse et al., 2023).

168 The conservation of *A. simplex* stable isotope values from previous hosts could reveal new  
169 insights into the diet and feeding range of the current host. For example, the spread in *A.*  
170 *simplex*  $\delta^{15}\text{N}$  values observed here (see SD in Figure 1 and full dataset in Data Supplement)  
171 could indicate the range of trophic levels of the various infected prey consumed by the host,

172 as each individual parasite might directly reflect the  $\delta^{15}\text{N}$  value of their former intermediate  
173 host e.g., zooplankton or small fish. This could make the parasite a natural proxy for  
174 understanding the trophic ecology of paratenic hosts. Additionally, the conservation of *A.*  
175 *simplex*  $\delta^{13}\text{C}$  values could suggest the geographic location of feeding, helping to assess  
176 whether the fish host's habitat range spans coastal, pelagic, or benthic zones, thereby  
177 providing a broader understanding of its ecological range and movement patterns. Further,  
178 research in this area could yield valuable insights into the host's diet and allow this  
179 hypothesis to be tested against other fish species.

180 Growth of *A. simplex* within fish has previously been postulated (Strømnes & Andersen,  
181 2003). This is discordant with our results, as it is unlikely that growth would occur without  
182 nutrient uptake. However, another hypothesis suggested by the same authors may be valid - it  
183 is possible that, as fish increase in size with age, and feed on prey from higher trophic levels,  
184 they accumulate larger *A. simplex* specimens that have had time to grow in an older prey. The  
185 larger parasites would then accumulate in the paratenic host, and as the host continues  
186 growing, older and smaller *A. simplex* could die off (~ 3-year life-span according to Smith  
187 (1983)), creating an accumulation of larger *A. simplex* leading to the impression of parasite  
188 growth. Sampling various sizes of *A. simplex* from a sedentary fish host (to avoid stable  
189 isotope baseline variations) and analysing  $\delta^{15}\text{N}$  values to determine if they correlate with host  
190 size would be one way to test this hypothesis.

191 If this hypothesis holds true, then the absence of nutrient transfer from host to parasite could  
192 explain why *A. simplex* does not actively seek to migrate beyond the body cavity. Indeed, in  
193 *M. merluccius* the majority of parasites were found free-floating in the body cavity with  
194 fewer encapsulated on the fish organs. It might also be possible that these encapsulations are  
195 incidental, following a host's immune response to the parasite's contact with vital host organs  
196 (Levsen et al., 2022). Once *A. simplex* has reached its final host, the warmer temperatures in

197 the marine mammal stomach prompt the L3 larvae to resume feeding and growth, preparing  
198 to transform into L4 and continue their life cycle through to reproduction. Indeed, adult *A.*  
199 *simplex* found in porpoise stomach harboured similar  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values to those of their  
200 host, indicative of an absorptive feeding nematode (Riekenberg et al., 2021).

201 Overall, this study has provided new information on nutritional requirements and metabolic  
202 activities of *A. simplex* parasites during their developmental stages, highlighting the  
203 importance of considering the developmental stage of a parasite when using stable isotopes to  
204 study parasitology. Finally, these findings improve our understanding of the *A. simplex* life  
205 cycle and ecological interactions, raising an interesting question: if *A. simplex* does not feed  
206 in their paratenic host fish, can this life-stage still be considered parasitic?

#### 207 **Authors contributions**

208 AJMS: Designed the study, prepared the samples for stable isotope measurements, analysed  
209 the data and wrote the first manuscript. O.B., M.G. and M.D: conducted sample gathering.  
210 HSL: Co-designed the study, contributed intellectual input, reviewed and edited the  
211 manuscript. MG: contributed intellectual input, reviewed and edited the manuscript. MD:  
212 contributed intellectual input, reviewed and edited the manuscript. SJB assisted with stable  
213 isotope analysis and edited the manuscript. JD analysed and processed the stable isotope data.  
214 All the authors approved the last version of the manuscript.

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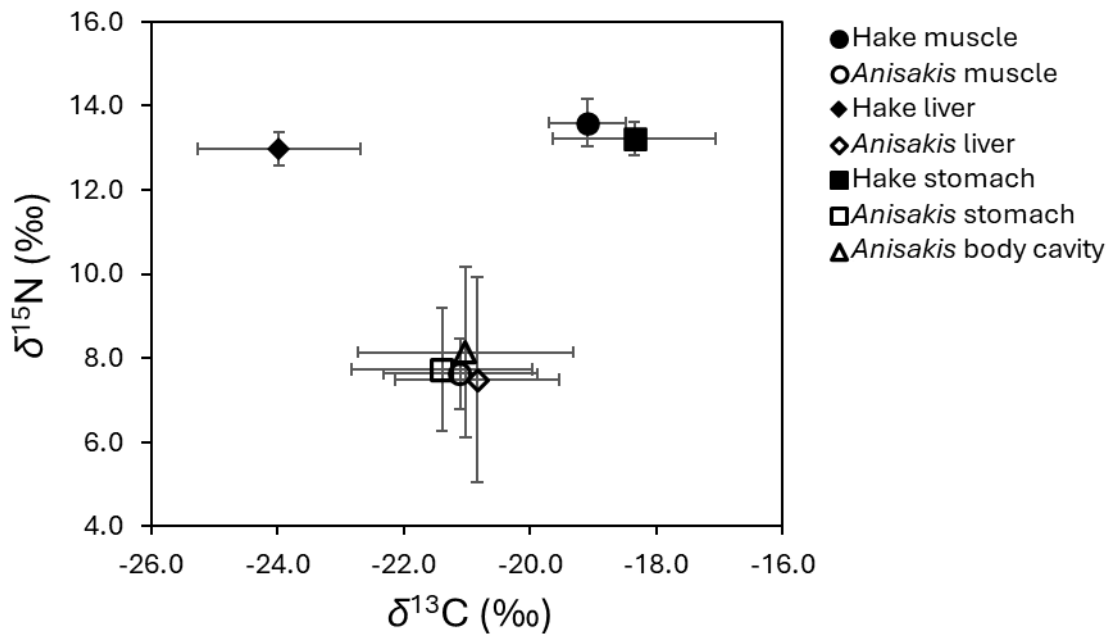
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390 **Figure**



391

392 Figure 1. Stable isotope biplot of  $\delta^{13}\text{C}$  versus  $\delta^{15}\text{N}$  values. Error bars represent standard

393 deviation of the mean values provided in Table 1. Filled symbols represent host tissues

394 (*Merlucciu merluccius*) and open symbols represent the parasite tissues (*Anisakis simplex*).

395 **Tables**

396 Table 1. Mean stable isotope values and standard deviation (SD), elemental composition (%N and %C), and C/N mass ratios of *Anisakis*

397 *simplex* and host *Merlucciu merluccius* fish tissues. The table also includes differences between stable isotope values of the host and parasite.

<b>Organism</b>	<b>Fish tissue</b>	<b>%N</b>	<b>Avg. <math>\delta^{15}\text{N}</math> (‰)</b>	<b>SD</b>	<b><math>\Delta^{15}\text{N}_{\text{hake-ani}}</math></b>	<b>%C</b>	<b><math>\delta^{13}\text{C}</math> (‰)</b>	<b>SD</b>	<b><math>\Delta^{13}\text{C}_{\text{hake-ani}}</math></b>	<b>C/N Ratio</b>
Hake	Muscle	13.92	13.60	0.55	5.97	44.27	-19.09	0.61	2.02	3.19
<i>Anisakis</i>		5.88	7.63	0.83		40.66	-21.11	1.22		6.98
Hake	Liver	4.96	12.97	0.39	5.49	59.47	-23.97	1.29	-3.14	13.72
<i>Anisakis</i>		5.94	7.48	2.45		40.83	-20.84	1.29		6.91
Hake	Stomach	13.02	13.22	0.30	5.49	42.41	-18.35	0.71	3.04	3.26
<i>Anisakis</i>		6.13	7.73	1.46		40.33	-21.39	1.44		6.64
<i>Anisakis</i>	Body cavity	6.24	8.14	2.02		40.82	-21.02	1.71		6.59
Avg. <i>Anisakis</i>		6.05	7.74		5.65	40.66	-21.09		0.64	6.78
SD <i>Anisakis</i>		0.17	0.28		0.28	0.24	0.23		3.31	0.19

398