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Stable Isotope Dynamics of Herbivorous Reef Fishes and Their Ectoparasites

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Abstract: Acanthurids (surgeonfishes) are an abundant and diverse group of herbivorous fishes on coral reefs. While their contribution to trophic linkages and dynamics in coral reef systems has received considerable attention, the role of linkages involving their parasites has not. As both consumers of fish tissue and prey to microcarnivores, external parasites may play a significant role in trophic transfer between primary consumers (and hence their predominantly algae-based diet) and the broader coral reef community. Stable isotope analysis is a common tool for studying trophic linkages which can be used for studies involving parasites. We examined the stable isotope ecology (^{13}C and ^{15}N) of copepod (*Caligus atromaculatus*) and monogenean (*Neobenedenia* sp.) ectoparasites collected from two species of Caribbean acanthurids (*Acanthurus coeruleus* and *Acanthurus bahianus*). There were significant intraspecific differences in isotope discrimination factors between parasites collected from the two different host species as well as interspecific differences between parasites collected from the same host species. Discrimination factors for ^{15}N were consistently positive but varied in magnitude depending on host and parasite species and were slightly lower than what would be expected for consumers. The ^{13}C discrimination factors for both monogeneans and copepods collected from *A. coeruleus* were consistently positive but were negative for copepods collected from *A. bahianus*. These findings emphasize the complexity of the stable isotope trophic interactions occurring between parasites and their hosts, highlighting the value of these types of host-parasite isotopic studies.

Keywords: Surgeonfish; Acanthuridae; coral reefs; ectoparasites; *Caligus*; *Neobenedenia*; stable isotope ecology; food webs; nanoEA

1. Introduction

Near-shore scleractinian coral reefs harbor the greatest biodiversity in the world's oceans [1–3]. This high biodiversity contained within a relatively small area facilitates an unparalleled network of complex interactions involving the biotic and abiotic community [4]. As with any ecosystem, a functioning coral reef system depends on and is structured by activities of its component trophic groups and its integrity is maintained via the flow of energy amongst these groups [5,6]. Thus, understanding the trophic interconnectivity of key trophic groups is essential for unravelling complex coral reef ecosystems.

An emerging modern view of ecology centers around the idea that the role of both parasites and micropredators in ecological communities has been greatly underestimated [7–10] and recent outbreaks of disease and parasite infestations in coral reef systems have further stimulated research into their role in coral reef energy dynamics. Parasites comprise the majority of biodiversity on coral reefs [3,11–14] and results to date indicate that these organisms are capable of modifying and directly or indirectly controlling the flow of energy in food webs and affecting host populations [10,15–17]. This, paired with their high abundance, biomass, and diversity, leads to the logical conclusion that they are ecologically significant organisms whose impacts extend to the community and ecosystem levels [15,18–20]. In some estuarine systems the overall biomass of parasites surpasses that of apex predators [21,22]. It is therefore surprising that in most food-web studies, parasites are often overlooked as key components of food-web energy dynamics [15,16,23,24]. This omission is largely due to their relatively small size and cryptic nature. The addition of parasites to food web models can increase connectance [25], food chain length [8,26], and overall species diversity [27]. Parasites can also indirectly alter energy flow by increasing the susceptibility of infected hosts to predation (e.g., [28]). Due to their intimate connection to hosts, parasites are often not considered to be readily available prey items. However, Johnson et al. [10] suggest that they are not only critical to energy transfer as consumers, but also as prey items acquired through concomitant predation, grooming/cleaning, or during free-living stages.

Herbivorous reef fishes are among the best studied trophic groups in coral reef systems [29–31]. They have major direct and indirect impacts on reef trophic dynamics via their role as grazers and primary consumers, serving as prey for other species, and in some cases through territorial behavior [32–37]. It is likely that given the diversity and abundance of parasites that they host, herbivorous fishes may also be influencing the trophic dynamics of reef ecosystems in other, more discrete ways. While their contribution to reef trophic dynamics through herbivory and as a prey source for piscivorous fishes has been well-studied, the role of parasites in trophodynamics of these fishes has been ignored.

Surgeonfishes (Acanthuridae) are among the most diverse and abundant herbivorous fishes in coral reef systems [38]. Although they are known to be primarily "herbivores," they exhibit significant interspecific variability within their diet. This includes selectively feeding on different types of algae and incidents of omnivory through ingestion of invertebrates while grazing on algae [39–41]. They are also known to harbor multiple internal and external parasites [42–45] which, given the high biomass of surgeonfishes, could contribute significantly to the energy transfer, to parasite consumers and coral reef communities at large. For example, surgeonfishes are frequent visitors to cleaning stations, where external gnathiid isopods, copepods, and monogeneans are eaten by cleaners [46–48]. These trophic interactions may represent a significant transfer of algal-derived biomass (in terms of carbon) between primary consumers and reef cleaning organisms.

Stable isotope analysis (SIA) has been used to help assess the links and magnitude of energy flow between organisms, which are the foundational components of food-web modeling [49,50]. In typical predator/prey interactions, carbon stable isotopes reflect primary carbon sources [51,52] and nitrogen stable isotopes are used to infer relative trophic levels among consumer groups [52–54]. Parasites and micropredators, however, employ a wide variety of unique feeding and life history strategies, and as a result have evolved complex metabolic processes for the digestion and assimilation of their diets [55]. It is not surprising that the limited (yet growing) number of studies focused on the stable isotope ecology of these organisms are finding that the classic isotopic patterns in carbon and nitrogen cannot be as easily applied to these cryptic yet ecologically significant organisms [56–64].

A significant source of variation in the isotopic values between parasites and "traditional" predators, as well as among different parasite species, is the site of attachment and the specific host tissue and/or fluids consumed by the parasites. Broadly, parasites fall into two groups, based on site of attachment: endoparasites and ectoparasites. Endo, or internal, parasites, including certain nematodes and helminths, live inside the host and typically enter the host via consumption, whereas ectoparasites

(e.g., ticks, fleas, and leeches) attach to the outside of the host. Two of the most common ectoparasites in marine systems are monogeneans and parasitic copepods.

Monogeneans are parasitic flatworms (Platyhelminthes: Monogenea) that are found attached to the fins, skin, and gills of a wide variety of fish, and feed on blood or mucus, depending on attachment site [65,66]. These parasites can have detrimental impacts on the health of both wild and captive fish populations [67,68] and may be an important dietary source for reef associated cleaning organisms [47,69–71]. Despite their ecological and economic significance, the only information available on the stable isotope ecology of parasitic monogeneans is for a single freshwater species [72] which is found attached to host fish gill tissues, feeding on blood [73]. The monogeneans were enriched relative to their host fishes: ^{13}C increased by up to 0.22‰ whereas ^{15}N increased by ~2‰, consistent with expectations for typical consumers relative to their diet.

In contrast to monogeneans, copepods are a more diverse group, with only about 1/3 of the species functioning as parasites/micropredators [74]. However, they infect a wide range of fish hosts, with significant impacts on individuals and populations [75] and are also likely to be significant dietary components for reef cleaner organisms [71]. Available data on the stable isotope ecology of parasitic copepods is also limited, with only three studies of note [56,57,76] where they quantified isotope discrimination, which is the difference in the isotopic ratio (^{13}C, ^{15}N) between the parasite and its host. Results from these preliminary studies indicate a surprising pattern of the copepods, exhibiting near consistent depletion in both ^{13}C and ^{15}N relative to host tissues (gill, muscle, skin, and eye) with a wide range of reported values for both. Interspecific differences in feeding strategies and attachment site of the parasites as well as interspecific difference in host physiology are speculated to be the cause of these somewhat unusual patterns of isotope fractionation [56,57,76] but there remains a significant amount of ambiguity surrounding the driving forces behind these patterns.

This study was inspired by the absence of data on the role of parasites in the trophic dynamics of surgeonfishes. Given the unusual patterns of stable isotope discrimination that have been documented for parasites and micropredators at large, the focus of this study was to determine if the carbon and nitrogen stable isotope discrimination patterns of a common eastern Caribbean monogenean, *Neobenedenia* sp. and parasitic copepod *Caligus atromaculatus* infecting two common, congeneric, Caribbean surgeonfishes (Acanthuridae) follow the patterns expected based on previous studies for these types of parasites.

2. Materials and Methods

The capsalid monogeneans of *Neobenedenia* sp. are some of the more notorious monogeneans due to their wide geographic and host range, infecting multiple species of hosts when most monogeneans tend to be host species specific [77]. Typical of monogeneans, oncomiracidia larvae hatch from eggs deposited on the substrate and attach to the host, where they remain for life [78,79]. While some monogeneans attach to the gills, *Neobenedenia* and other capsalids attach to the body and fins of the host. In the eastern Caribbean, *Neobenedenia* have been found in low numbers on a wide range of fish species [80,81] but are particularly common on surgeonfishes (Acanthuridae), especially the blue tang (*Acanthurus bahianus*) [43,82]. They are also eaten by cleaner shrimps [47].

Copepods of the genus *Caligus* exhibit little host specificity parasitizing a wide range of host fish families [83]. As adults these copepods are exclusively parasitic, generally utilizing a single host during its life cycle [84] but there is increasing evidence that some species may parasitize multiple hosts [85]. Little information is available for *C. atromaculatus* but they are primarily found on the skin of host fish and are believed to consume host skin tissue, mucus, and/or blood [86,87].

Two species of Acanthurids were selected as hosts for this study. The blue tang, *Acanthurus coeruleus*, and the ocean surgeonfish, *Acanthurus bahianus*. These herbivorous fishes are common in coral reefs and adjacent habitats throughout the Caribbean region [88]. Both are hosts to *C. atromaculatus* and *N. melleni*, and are common at our study sites [43]. Although closely related species, *A. coeruleus* and *A. bahianus* appear to have significant differences in terms of both feeding ecologies and digestive

physiology [39]. This provided an opportunity to examine any differences in patterns of stable isotope discrimination between two different species of parasite collected from the same species of host, and to compare differences between hosts. Blood was selected for analysis of host stable isotopes as it could be collected without sacrificing fish and is one of the presumed dietary sources for both species of parasites studied.

Sampling was conducted from June-August 2012, 2013, and 2018 at Greater Lameshur Bay (GLB), St. John, U.S. Virgin Islands (USVI, 18°19′ N, 65°44′ W), and Brewers Bay (BRB), St. Thomas, U.S. Virgin Islands (Figure 1). Fish hosts were collected at night using hand nets and flashlights with SCUBA or by snorkeling. Once netted, fish were carefully transferred to holding tanks prior to processing and were assigned a unique fish host ID which was used to pair tissue and parasite samples collected from each individual host fish. Parasites were collected by administering freshwater baths to host [43] and then sieving the contents of the bath to collect the parasites. Adult monogeneans and adult copepods were sorted from the contents of the sieve under a dissecting scope and labeled with their corresponding fish ID and preserved in 80% ethanol. Ethanol has been determined to be an adequate preservation method for parasites with no significant preservative associated artifacts in stable isotope analysis for a variety of invertebrate organisms [60,89–91]. While we did not specifically test for ethanol preservation effects on isotope values for these specific parasites, based on studies of similar organisms that found no significant change in isotope values with ethanol preservation, ethanol artifacts were considered negligible in the current study. Host fish which harbored a sufficient number of parasites for isotope analysis were sampled for blood (non-lethal) using an insulin needle and syringe via the caudal or dorsal aortas. Blood samples were immediately dried at 50–60 °C for 24–36 h. Once completely dried, samples were stored in airtight cryovials and packed with desiccant for transport. Monogeneans were only analyzed for *A. coeruleus* due to a lack of sufficient numbers of monogeneans collected from *A. bahianus*. This work was completed under Arkansas State University IACUC number 778227-1.

Parasites and host blood were processed for stable isotope analysis according to methods described in Demopoulos and Sikkel [60] and Jenkins et al. [61]. In order to meet the minimum and maximum mass requirements for accurate stable isotope analysis, individual parasites from the same host were either pooled (ranging from 2–11 individuals) or subsampled. Subsampling of individual parasites was done under the microscope using a scalpel and care was taken to split individuals along their axis of symmetry to ensure an appropriate subsample. Parasite mass was recorded after drying samples. For pooled parasite samples with more than one individual, the average mass of the pooled individuals was used as a proxy. Prior to analysis, copepod samples were acidified using 10% PtCl to remove inorganic carbon associated with their chitinous exoskeleton [92].

Two elemental analyzers were used to collect stable isotope data for the samples used in this study. Host blood and a subset of the parasite samples (large individual copepods and pooled monogeneans when sample sizes permitted) were analyzed at Washington State University, WA, using a Costech (Valencia, CA, USA) elemental analyzer interfaced to a GV Instruments Ltd (Manchester, UK) Isoprime^{TM} isotope ratio mass spectrometer. The remaining samples were analyzed at the University of California, Santa Cruz using an automated "nanoEA" trapping system [93], which combines a modified Carlo Erba CE1108 Elemental Analyzer and Thermo Electron Gas Bench II to cryofocus small samples (1–8 mg C) in order to enhance signal to noise. Reproducibility of the analysis and consistency of data between the two mass spectrometers was confirmed using a bovine liver standard [49] within ± 0.2‰ for both δ^{13}C and δ^{15}N on both elemental analyzers used. Dual ^{13}C and ^{15}N analysis for samples was not always possible due to limitations in machine sensitivity and/or machine error so sample sizes vary between carbon and nitrogen data (see Supplemental Table S1).

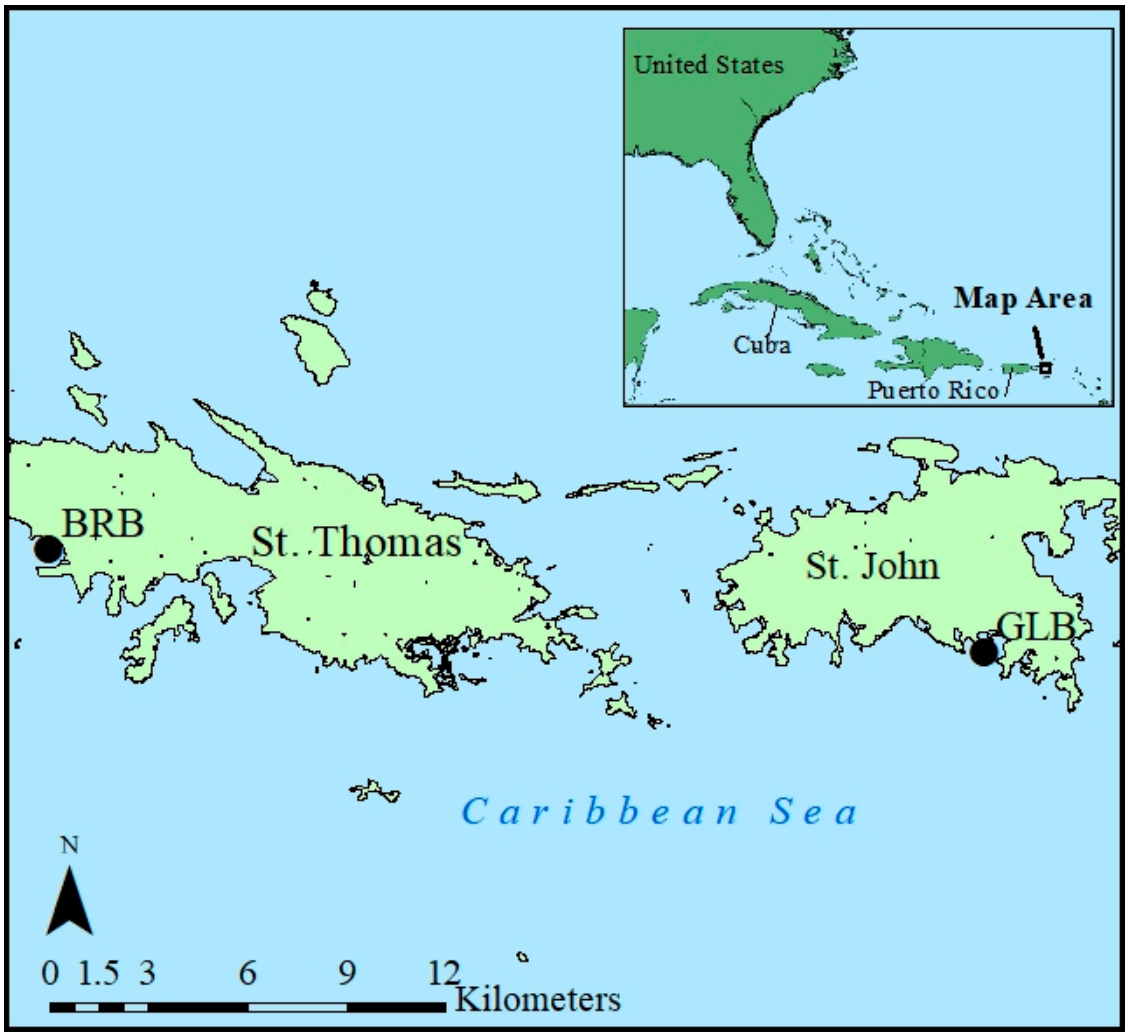

Figure 1. Location of study sites Brewers Bay (BRB) and Greater Lameshur Bay (GLB) where host fish and parasites were collected.

Stable isotope ratios are expressed as δ values, in units of per mil (‰), using the following equation:

$$\delta^{13}C \text{ or } \delta^{15}N = ([R_{sample}/R_{standard}] - 1) \times 10^3 \quad (1)$$

where R is the ratio of $^{13}C/^{12}C$ or $^{15}N/^{14}N$ and standards are PeeDee Belemnite and atmospheric nitrogen gas for carbon and nitrogen, respectively. Data were examined for potential lipid contribution based on the C:N data and we found no indication of a lipid effect across samples [94], so we did not lipid-correct the isotope data for any of the taxa.

Isotopic discrimination factors between paired samples (i.e., between copepods and blood from their respective host) are expressed as Δ values, in units of per mil (‰), using the following equation:

For parasite – host blood discrimination:

$$\Delta^{13}C \text{ or } \Delta^{15}N = (\delta_{parasite} - \delta_{host\ blood}) \quad (2)$$

Linear mixed effect (LME) modeling was used to test for isotopic differences between host tissues and associated paired parasites. By including the unique host fish IDs as a random effect, LME modeling accounts for instances of pseudoreplication, which arose from having replicates of parasites collected from the same fish host. LME models were fit using the restricted likelihood ratio estimation

method as part of the lmer function of the R package *lme4* [95]. All data analyses were conducted using the R statistical program [96].

Model selection was determined using a type-II (hierarchical) analysis of variance (Wald X^2) on the initial model to test for significance of the candidate fixed effects and interactions using the *car* package in R [97]. Significant predictors (Table 1 for a list of factors tested), identified through the Wald X^2 analysis of variance, were then refit using the maximum likelihood estimation method (ML), and likelihood ratio test was used to confirm significance of model relative to a null model (also using ML) fitted with only individual host fish ID as a random intercept to account for instances of pseudo-replication. Preliminary comparisons (Students t-test) of data from the two sites indicated no significant variations in stable isotopes of host fish or parasites between the two sites (i.e., $p > 0.05$), therefore results from both sites were pooled for all comparisons. The proportion of variance in the observed data that was explained by the fixed and random effects was calculated based on marginal and conditional R^2 ($R^2_{GLMM(m)}$ and $R^2_{GLMM(c)}$) [98] using the r.squaredGLMM function from the *MuMIn* package in R [99]. The validity of the assumptions of linearity, homoscedasticity, and normality were assessed using fitted vs. residual, scale-location, and Q-Q diagnostic plots. Significance of isotope discrimination factors and pairwise means comparisons were assessed with model estimated marginal means and associated 95% confidence intervals generated using the package *emmmeans* [100] (Tables S2 and S3). Additionally, we conducted a one-way ANOVA to identify if time was a factor explaining the isotopic variance for a subset of the samples (*A. coeruleus* blood, copepods, and monogeneans). These were selected because they had sufficient sample sizes at all collection times. We compared C:N ratios of host blood with parasite-host discrimination factors using Pearson correlation. Differences in C:N ratios of host blood across sampling years were assessed with a Welch one-way test for *A. coeruleus* and a Wilcoxon rank-sum test for *A. bahianus*. Lastly, a one-way analysis of variance of *A. coeruleus* blood (insufficient sample size for *A. bahianus*) vs. sample month was conducted.

Table 1. Results from type-II (hierarchical) analysis-of variance (Wald X^2) on full models which included all factors of interest. Factors which were deemed significant (*p*-values in bold) were fitted to the final model.

Response Variable	Variables Tested	X^2	df	*p*-Value
δ^{13}C	Sample type (blood, monogenean, copepod)	70.7032	2	**<0.001**
	Host Species (*A. coeruleus*, *A. bahianus*)	1.9597	1	0.162
	Sample type X host species	24.6094	1	**<0.001**
δ^{15}N	Sample type (blood, monogenean, copepod)	16.206	2	**<0.001**
	Host Species (*A. coeruleus*, *A. bahianus*)	6.3899	1	**0.011**
	Sample type X host species	0.4252	1	0.514
Δ^{13}C parasite-host blood	Parasite type (monogenean, copepod)	25.071	1	**<0.001**
	Host Species (*A. coeruleus*, *A. bahianus*)	18.68	1	**<0.002**
	Parasite mass	0.036	1	0.849
	Host blood δ^{13}C	2.751	1	0.097
	Host species X parasite mass	0.85	1	0.357
	Parasite type X parasite mass	8.917	1	**0.003**
	Host Species X host blood δ^{13}C	1.811	1	0.178
	Parasite type X host blood δ^{13}C	0.4	1	0.527
Δ^{15}N parasite-host blood	Parasite type (monogenean, copepod)	0.247	1	0.619
	Host Species (*A. coeruleus*, *A. bahianus*)	8.106	1	**0.004**
	Parasite mass	10.855	1	**0.001**
	Host blood δ^{15}N	0.641	1	0.423
	Host species X parasite mass	0.792	1	0.373
	Parasite type X parasite mass	6.577	1	**0.010**
	Host Species X host blood δ^{15}N	1.928	1	0.165
	Parasite type X host blood δ^{15}N	11.769	1	**0.001**

3. Results

3.1. Carbon Stable Isotopes

Acanthurus coeruleus blood ($\delta^{13}C = -15.3 \pm 0.1‰$) was slightly depleted relative to *A. bahianus* ($\delta^{13}C = -14.6 \pm 0.2‰$, Figure 2). Monogeneans collected from *A. coeruleus* were enriched relative to *A. coeruleus* blood ($\delta^{13}C = -13.7 \pm 0.2‰$) and had an average $\Delta^{13}C$ of 1.9 ± 0.1‰ (Table 2) for parasite-host pairs. We examined the variation in $\delta^{13}C$ data for the monogeneans relative to *A. coeruleus* blood (Supplemental Figure S1a) and found low variation among parasite $\delta^{13}C$ values across fish hosts. Copepods collected from *A. coeruleus* were also enriched in ^{13}C relative to host blood ($\delta^{13}C = -14.4 \pm 0.2‰$, Figure 2) and had an average $\Delta^{13}C$ discrimination factor of 1.0 ± 0.2‰. In contrast to the positive $\Delta^{13}C$ discrimination factor observed for copepods feeding on *A. coeruleus*, copepods collected from *A. bahianus* were consistently depleted in ^{13}C relative to host blood ($\delta^{13}C = -15.3 \pm 0.3‰$, Figure 2) with an average parasite-host $\Delta^{13}C$ of −0.6 ± 0.1‰.

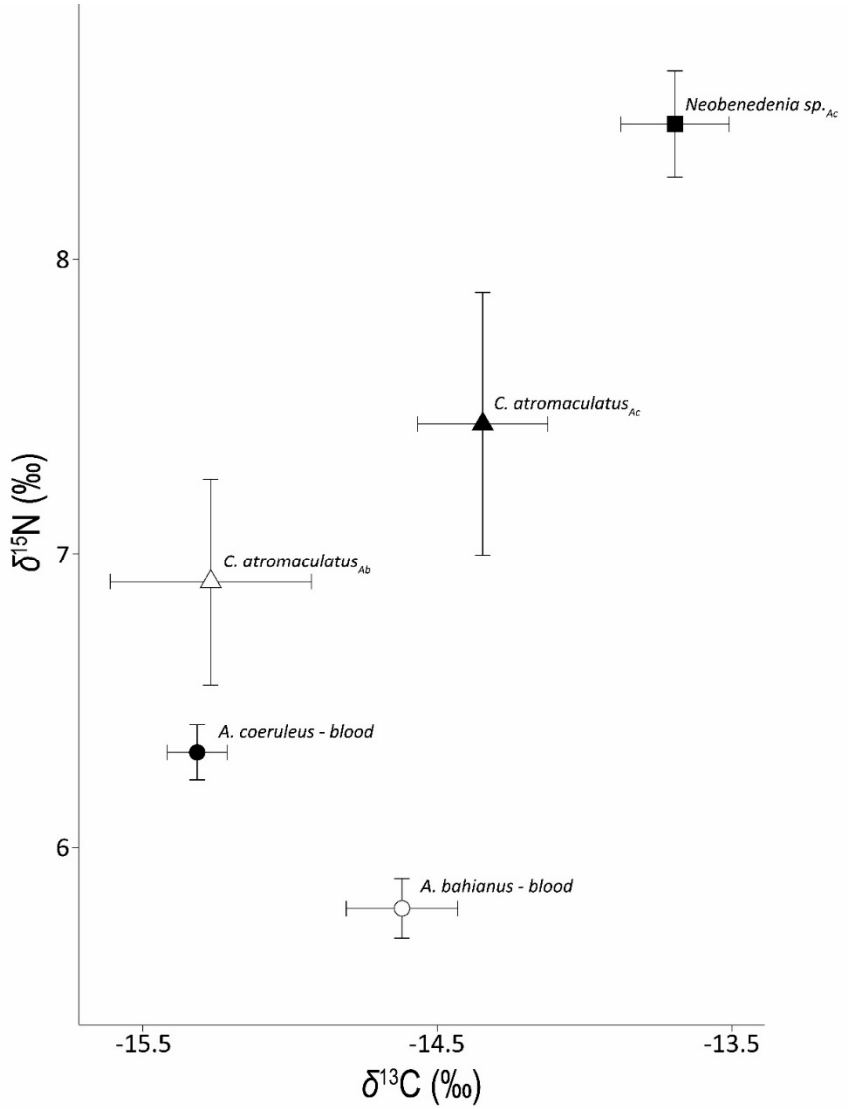

Figure 2. Mean ± SE $\delta^{13}C$ and $\delta^{15}N$ data for host fish blood (circles) and associated *Caligus atromaculatus* (triangles) and *Neobenedenia* sp. (square) collected from *A. coeruleus* (*Ac*) and *A. bahianus* (*Ab*) hosts.

Table 2. Summary of stable isotope data (‰, mean ± SE, range in parentheses), discrimination factors ($\Delta^{13}C$, $\Delta^{15}N$), carbon to nitrogen ratios (C:N), and sample sizes (*n*) for host fish blood and parasites.

Host	$\delta^{13}C$	$\delta^{15}N$	C:N	Parasite	$\delta^{13}C$	$\Delta^{13}C$	$\delta^{15}N$	$\Delta^{15}N$	C:N
A. coeruleus	−15.3 ± 0.1 (−17.4 to −13.5, *n* = 63)	6.3 ± 0.1 (4.5 to 7.9, *n* = 64)	3.7 ± 0.0 (3.3 to 4.4, *n* = 64)	*C. atromaculatus*	−14.4 ± 0.2 (−16.3 to −12.2, *n* = 23)	1.0 ± 0.2 (−0.2 to 3.7, *n* = 22)	7.4 ± 0.4 (3.1 to 10.9, *n* = 24)	0.9 ± 0.4 (−4.5 to 4.2, *n* = 23)	3.0 ± 0.1 (2.5 to 3.4, *n* = 14)
				Neobenedenia sp.	−13.7 ± 0.2 (−17.1 to −10.5, *n* = 59)	1.9 ± 0.1 (0.6 to 3.4, *n* = 44)	8.5 ± 0.2 (4.2 to 12.6, *n* = 85)	2.0 ± 0.1 (−0.1 to 5.2, *n* = 70)	3.3 ± 0.0 (3.1 to 3.8, *n* = 18)
A. bahianus	−14.6 ± 0.2 (−16.3 to −12.4, *n* = 33)	5.8 ± 0.1 (4.7 to 7.0, *n* = 31)	3.7 ± 0.0 (3.5 to 4.0, *n* = 33)	*C. atromaculatus*	−15.3 ± 0.3 (−17.9 to −12.2, *n* = 21)	−0.6 ± 0.1 (−1.8 to 0.1, *n* = 15)	6.9 ± 0.3 (3.4 to 10.0, *n* = 22)	1.4 ± 0.3 (−1.0 to 3.6, *n* = 17)	3.1 ± 0.2 (2.47 to 4.0, *n* = 6)

Host species and an interaction between host species and sample type (host blood, monogenean, copepod) accounted for a significant amount of the observed variance in $\delta^{13}C$ (R^2_m/R^2_c = 0.24/0.66) when fitted to a model with individual host "Host ID" as a random effect (Table 3). Both parasite type and host species appeared to be significant predictors of parasite-host $\Delta^{13}C$ and there was a significant negative relationship between the parasite-host $\Delta^{13}C$ and parasite mass for monogeneans (Table 3).

Table 3. Model estimated coefficients.

Response Variable	Predictor Variable	Estimate ± SE	t-Value	*p*-Value
$\delta^{13}C$	(Intercept)	−15.4 ± 0.2	−93.579	<0.001
	C. atromaculatus	0.9 ± 0.2	3.982	0.009
	Neobenedenia sp.	1.6 ± 0.2	9.103	0.002
	A. bahianus	0.9 ± 0.3	3.214	0.004
	C. atromaculatus X *A. bahianus*	−1.6 ± 0.3	−4.961	<0.001
$\delta^{15}N$	(Intercept)	6.7 ± 0.4	18.213	<0.001
	C. atromaculatus	0.6 ± 0.5	1.344	0.197
	Neobenedenia sp.	1.4 ± 0.4	3.467	0.003
	A. bahianus	−0.8 ± 0.3	−2.524	0.013
$\Delta^{13}C$	(Intercept)	−2.1 ± 1.5	−1.412	0.164
	A. bahianus	−1.5 ± 0.3	−5.075	<0.001
	Neobenedenia sp.	1.5 ± 0.4	4.073	0.001
	Host blood $\delta^{13}C$	−0.2 ± 0.1	−1.792	0.08
	Parasite mass	34.7 ± 15.6	2.226	0.029
	Neobenedenia sp. X Parasite mass	−40.3 ± 18.6	−2.168	0.037
$\Delta^{15}N$	(Intercept)	3.5 ± 1.6	2.161	0.033
	A. bahianus	0.2 ± 0.4	0.379	0.705
	Neobenedenia sp.	−3 ± 2	−1.55	0.124
	Parasite mass	80.7 ± 19.4	4.152	<0.001
	Host blood $\delta^{15}N$	−0.6 ± 0.2	−2.37	0.02
	Neobenedenia sp. X Parasite mass	−70.6 ± 25	−2.825	0.006
	Neobenedenia sp. X Host blood $\delta^{15}N$	0.8 ± 0.3	2.542	0.013

Model estimates indicated no significant difference in blood $\delta^{13}C$ between the two species of hosts (p = 0.97), however estimates for copepods indicated a significant difference depending on host species (p = 0.04). Estimated 95% confidence intervals indicated that the $\Delta^{13}C$ discrimination factors for copepods and monogeneans collected from *A. coeruleus* and copepods collected from *A. bahianus* were all significant (i.e., do not span zero, Table S3). In addition, the estimated $\Delta^{13}C$ means for the different parasites indicate significantly different $\Delta^{13}C$ discrimination factors associated with each of the different groups of parasites (monogeneans from *A. coeruleus*, copepods from *A. coeruleus*, and copepods from *A. bahianus*, Table S2).

3.2. Nitrogen Stable Isotopes

Acanthurus coeruleus blood ($\delta^{15}N$ = 6.3 ± 0.1‰) was enriched in ^{15}N relative to *A. bahianus* blood ($\delta^{15}N$ = 5.8 ± 0.1‰, Figure 2). Both monogeneans ($\delta^{15}N$ = 8.5 ± 0.2‰) and copepod parasites ($\delta^{15}N$ = 7.4 ± 0.4‰) collected from *A. coeruleus* were enriched in ^{15}N relative to host blood (Figure 2) with average parasite-host $\Delta^{15}N$ discrimination factors of 2.0 ± 0.1‰ and 0.9 ± 0.4‰, respectively. We examined the variation in $\delta^{15}N$ data for the monogeneans relative to *A. coeruleus* blood (Supplemental Figure S1b) and found high variation among parasites across individual fish hosts. Copepods collected from *A. bahianus* hosts ($\delta^{15}N$ = 6.9 ± 0.3‰) were enriched in ^{15}N relative to *A. bahianus* blood ($\delta^{15}N$ = 5.8 ± 0.1‰, Figure 2) with an observed mean parasite-host $\Delta^{15}N$ discrimination factor of 1.4 ± 0.3‰.

Sample type and host species were both significant predictors of $\delta^{15}N$ and the magnitude of these effects was consistent across levels (i.e., no interactions, Table 1). For the final model, fixed effects alone accounted for 24% of the variance observed and this only increased to 25% when factoring in Host ID

as a random effect (R^2_m/R^2_c = 0.24/0.25). Parasite-host $\Delta^{15}N$ appeared to be significantly influenced by host species but not the type of parasite. Both host blood $\delta^{15}N$ and parasite mass were significant predictors of Parasite-host $\Delta^{15}N$ but varied in effect depending on parasite type. Parasite mass had a positive effect on parasite-host $\Delta^{15}N$ for copepods but the opposite effect for monogeneans (Table 3). Parasite-host $\Delta^{15}N$ decreased with increasing host blood $\delta^{15}N$ for both copepods and monogeneans. However, the opposite relationship was indicated for monogeneans with parasite-host $\Delta^{15}N$ decreasing with increasing host blood $\delta^{15}N$ (Table 3). With the final model, fixed effects alone accounted for 18% of the observed variance and 46% when host ID was included as a random effect (R^2_m/R^2_c = 0.18/0.46).

Model estimates for $\delta^{15}N$ indicated the observed differences in host blood and copepod $\delta^{15}N$ between the two host species was not significant (p = 0.15 and 0.15, Table S2). Model generated CIs indicated significant positive $\Delta^{15}N$ discrimination factors for both parasite types regardless of host species (Table S3). Additionally, model estimates indicated a significant difference in parasite – host $\Delta^{15}N$ between the two species of parasites collected from *A. coeruleus* (p = 0.01) and no significant difference $\Delta^{15}N$ between copepods from the two species of hosts (p = 0.81, Table S2).

3.3. Stable Isotope Patterns over Time and with C:N

For *A. coeruleus* blood, there was no difference among years in $\delta^{13}C$ (p = 0.196, df = 2) or $\delta^{15}N$ (p = 0.235, df = 2). For copepods collected on *A. coeruleus*, there was also no difference among years for $\delta^{13}C$ (p = 0.31, df = 2), but there was a significant effect for $\delta^{15}N$ (p = 0.0316, df = 2), with 2018 having higher $\delta^{15}N$ than either 2013 or 2012. For collection month, *A. coeruleus* blood was not significantly different in $\delta^{13}C$ (p = 0.101, df = 2), but there were differences in $\delta^{15}N$ ($p < 0.001$, df = 2), with June having the highest (6.8 ± 0.1‰) and July the lowest (5.8 ± 0.2‰) values. Likewise, for monogeneans collected from *A. coeruleus*, $\delta^{13}C$ did not differ among months (p = 0.192, df = 2); however, for $\delta^{15}N$ significant differences among months were evident ($p < 0.001$, df = 2), with June having the highest (9.1 ± 0.3‰) and July the lowest (7.4 ± 0.2‰) values, mirroring the results from the host blood. For *A. coeruleus*-associated copepods, there were no significant differences among months for $\delta^{13}C$ (p = 0.839, df = 2) or $\delta^{15}N$ (June and July only, because there were insufficient sample sizes from August, t = −0.063, df = 19, p-value = 0.723). Due to sampling limitations, temporal analysis of *A. bahianus* blood was not possible because a majority of those samples were collected in July.

For *A. coeruleus*, we compared the discrimination factors for monogeneans relative to C:N data using Pearson's correlation tests, and found no significant difference in $\Delta^{13}C$ and host blood C:N (p = 0.936, df = 42, t-statistic = 0.080). However, there was a significant correlation between $\Delta^{15}N$ and host blood C:N (p = 0.019, df = 68, t-statistic = −2.41), with a weak correlation coefficient of −0.28. For copepods associated with *A. coeruleus*, there was no significant correlation for either $\Delta^{13}C$ (p = 0.626) or for $\Delta^{15}N$ (p = 0.768, df = 21, t-statistic = −0.299). Likewise, for *A. bahianus*, there was no significant correlation between C:N of host blood and copepod discrimination factors for $\Delta^{13}C$ (p = 0.870, df = 13, t-statistic = −0.166) or for $\Delta^{15}N$ (p = 0.948, df = 15, t-statistic = −0.066).

For both *A. coeruleus* and *A. bahianus* we compared host blood C:N values across the different sampling years. For *A. coeruleus*, a Welch one-way test indicated no significant difference between the three sampling years (F = 1.85, df = 2, p = 0.190). A Wilcoxon rank-sum test of *A. bahianus* host blood C:N indicated a significant difference (W = 54.5, p = 0.016) between 2012 ($C:N_{mean}$ = 3.8 ± 0.1, n = 4) and 2018 ($C:N_{mean}$ = 3.7 ± 0.0, n = 4). Due sample size limitations, we were only able to assess variation of C:N values across the sampling months for *A. coeruleus*. A one-way analysis of variance indicated significant differences between the sampling months ($C:N_{mean}$ = 3.6 ± 0.0 for June , July, and August; p = 0.001, df = 2). For both the year and month assessments the magnitude of all significant differences fell within the margin of error for our C:N (determined based on standard error for C:N of bovine liver standards run with parasite and fish tissue samples) data and were considered not significant.

4. Discussion

This study constitutes one of the most robust analyses of the stable isotope ecology of parasitic monogeneans and copepods from any fish species to date. While there is a significant lack of data regarding the stable isotope ecology of parasites in general, it is especially sparse for copepods and monogeneans. With one exception [63] previous studies on the stable isotope ecology of these parasites are limited to sample sizes ranging from 1–7 (see Table 4). This is not surprising given the exceptionally cryptic nature of these parasites compared to larger ectoparasites such as gnathiid and cymothoid isopods which have received more attention in recent studies [60,61,101,102].

The differences in direction of the parasite-host $\Delta^{13}C$ discrimination factor for the copepods is particularly interesting given the phylogenetic and ecological similarities between the two species of hosts [39]. *Caligus atromaculatus* copepods collected from *A. bahianus* were consistently depleted in ^{13}C relative to host blood, consistent with other studies of parasitic copepods [56,57,63] (see Table 4) as well as other terrestrial and marine micropredators and parasites [56–58,60,61,103]. On the other hand, the same species of copepod were significantly enriched in ^{13}C relative to *A. coeruleus* blood (Figure 2, Table 2) exhibiting typical patterns of ^{13}C fractionation associated with consumers relative to their prey [54,104]. Variations in $\Delta^{13}C$ discrimination factors between the same species of parasite feeding on different species of hosts is consistent with published results from parasitic copepods [57] and other species of fish parasites [61,72]. However, the shift in direction of parasite-host $\Delta^{13}C$, from positive for *A. coeruleus* to negative for *A. bahianus* for these copepods is unusual and may be the due, in part, to the physiological and dietary differences between two species of hosts [39].

The variation in stable isotope turnover rates among organisms is another factor to consider when resolving between-species differences in discrimination factors [105]. If the isotopic turnover rates for either of the parasites examined in this study are significantly slower than those for either species of host fish, their respective stable isotope signals may be representative of different time scales and not always at equilibrium. This would be especially relevant if there is temporal variation in the diet of the host as the parasite would retain a residual isotopic signal of previous diets longer than the hosts' blood. In the context of this study, such conditions might provide a reasonable explanation for the differences ^{13}C discrimination between *C. atromaculatus* from the two host species if there is temporal variability in the diet of *A. coeruleus* or *A. bahianus*. For *A. coeruleus* blood, ^{13}C values were consistent over time, indicating that these fish are either feeding on consistent food sources and/or their sources of carbon have consistent isotope values over time. It is possible that variations in food selection for *A. bahianus* may exist outside of this timescale, but this would require future studies to resolve. Thus, it is difficult to speculate on the possibility of shift in diet for the host fish occurring outside of the timescales examined in this study without knowing more about the isotopic turnover rates for *A. coeruleus* and *A. bahianus* and their associated parasites. There is a wide range of reported ^{13}C and ^{15}N turnover rates for fish blood [106–109], some exceeding the timescale of our study (June–August, 3 months total). To the best of our knowledge, turnover rates for either of our host species or acanthurids in general are unpublished. Furthermore, there do not appear to be any published data on turnover rates for copepods or monogeneans in general. In lieu of information on the species-specific discrimination rates we can speculate that given the short lifespan of *Neobenedenia* sp. [110] and the tight coupling between the *A. coeruleus* blood and monogenean isotope data that these parasites are recording the fish isotope composition over similar time scales. Controlled feeding experiments would be needed to resolve whether blood and monogeneans have similar turnover timescales. For *C. atromaculatus*, the life cycle of its congener, *C. rogercresseyi*, indicates that they may stay attached to the fish host up to 45 days [111], which may exceed typical turnover times for blood [106–109].

Table 4. Summary of data on parasite-host discrimination factors (‰) and sample sizes (*n*) for monogeneans and parasitic copepods. Data from this study are in bold. Samples sizes for present study are reported for both $\Delta^{13}C$ and $\Delta^{15}N$.

Host	Host Tissue	Parasite Type	Parasite Species	Family	*n*	$\Delta^{13}C$	$\Delta^{15}N$	Authors
Acanthurus coeruleus	**Blood**	**Monogenean**	*Neobenedenia* sp.	**Capsalidae**	**44, 70**	**1.9**	**2.0**	**Present study**
Labeobarbus aeneus	Muscle	Monogenean	*P. ichthyoxanthon*	Diplozoidae	7	0.09	2.05	Sures et al., 2018
Labeobarbus kimberleyensis	Muscle	Monogenean	*P. ichthyoxanthon*	Diplozoidae	2	0.22	2.31	Sures et al., 2018
Acanthurus coeruleus	**Blood**	**Copepod**	*Caligus atromaculatus*	**Caligidae**	**22, 23**	**1.0**	**0.9**	**Present study**
Acanthurus bahianus	**Blood**	**Copepod**	*Caligus atromaculatus*	**Caligidae**	**15, 17**	**−0.6**	**1.4**	**Present study**
Gadus morhua	Gill	Copepod	*Clavella adunca*	Lernaeopodidae	1	−2.19	−1.29	Deudero et al., 2002
Merlangius merlangus	Gill	Copepod	*Clavella adunca*	Lernaeopodidae	5	−4.06	−4.23	Deudero et al., 2002
Clarias gariepinus	Muscle	Copepod	*Lamproglena clariae*	Lernaeidae	44	−0.5	0.24	Gilbert et al., 2020
Platichthys flesus	Skin	Copepod	*Lepeophtheirus pectoralis*	Caligidae	2	0.11	−0.22	Deudero et al., 2002
Sprattus sprattus	Eye	Copepod	*Lernaeenicus sprattae*	Pennellidae	1	−1.39	0.60	Deudero et al., 2002
Gadus morhua	Gill	Copepod	*Lernaeocera branchialis*	Pennellidae	2	−2.39	−0.82	Deudero et al., 2002
Melanogrammus aeglefinus	Gill	Copepod	*Lernaeocera branchialis*	Pennellidae	3	−0.62	1.10	Deudero et al., 2002
Merlangius merlangus	Gill	Copepod	*Lernaeocera branchialis*	Pennellidae	6	−1.55	−2.61	Deudero et al., 2002
Platichthys flesus	Muscle	Copepod	*Lernaeocera branchialis*	Pennellidae	3	−1.63	−0.81	Pinnegar et al., 2001
Merlangius merlangus	Gill	Copepod	unknown	unknown	2	−1.97	−5.82	Deudero et al., 2002

Trophic discrimination factors, $\Delta^{15}N$, associated with the monogeneans from *A. coeruleus* hosts were consistent with previous published values from monogenean and fish host- stable isotopes (Table 4). Monogeneans in both the present study and that of Sures et al. [72] were enriched in ^{15}N (~2‰) relative to host tissue, consistent with ^{15}N trophic discrimination between a consumer and its diet [53] (e.g., a single trophic step). The copepods collected from both *A. coeruleus* and *A. bahianus* were similarly enriched (albeit to a lesser extent) in ^{15}N relative to blood from their respective host species, but their associated $\Delta^{15}N$ discrimination factors fall below the threshold of what is typically expected for consumers. The overall trend for other species of parasitic copepods (including another species of Caligidae) [57] appears to be negative ^{15}N discrimination with only few instances of positive discrimination relative to host blood (Table 4). This low discrimination of ^{15}N is consistent with observations for gnathiid isopods [60,61]. More efficient digestion and assimilation of blood is associated with less fractionation, and thus, lower discrimination, of ^{15}N compared to other host tissues [105].

Significant differences in the carbon stable isotope data between the copepods collected from *A. coeruleus* versus *A. bahianus* indicate that differences in host physiology may be playing a significant role in the turnover rates of ^{13}C between hosts and parasites. Isotopically speaking, the blood of *A. coeruleus* and *A. bahianus* do not appear significantly different (Figure 2, Table S2), however, they are known to have markedly different feeding habits and digestive physiology [39]. It is well documented that differences in diet, condition, and metabolic physiology can have significant impacts on the isotopic turnover rates between an organism and its diet [105,112,113] which could in turn impact the isotopic turnover rates of associated parasites. However, much of the work on the topic has focused on consumers and not parasites. Given their unique physiology and feeding ecology, it is unclear whether parasites would behave similarly without additional studies. The handful of studies which have examined stable isotope turnover rates for a single species of parasite infecting multiple host species indicate that there are likely host specific traits influencing the rate of isotope discrimination between a parasite and its host [57,61]. As such, it is possible that physiological differences between the two host species may be contributing to the differences in parasite – host $\Delta^{13}C$ between *C. atromaculatus* feeding on *A. coeruleus* versus *A. bahianus* and resolving physiological differences would require future studies.

One of the major challenges faced when trying to draw comparisons between parasite stable isotope studies is differences in tissues selected for studies (Table 4) due to the possibility that selected host tissues may not be an accurate representation of the parasites diet [56,57,60]. Blood was selected for this study because it is easy to collect from hosts with minimal loss of life and it is likely the primary food source for both species of parasites examined in this study [65,66,86,87]. This is not, however, definitive proof that these parasites were feeding on host blood which leaves open the possibility that the observed discrimination may be the result of a mismatch in actual isotopic values of the assumed dietary source (blood) and the true diet of the parasite (other host tissue). It should also be noted that comparisons between this study and others can be impacted by the difference in host tissues being analyzed and highlights the importance of careful consideration of which host tissues to analyze in parasite isotope studies [61]. We also note that while ethanol preservation effects on isotope values on crustaceans, including copepods, as well as soft bodied invertebrates, have been identified as negligible in other studies [60,114,115], future analysis examining the effects on monogeneans specifically should be conducted. Regardless, the emerging theme is that the driving forces behind the more complex patterns in stable isotope ecology associated with micropredators and parasites has been largely attributed to their complex life histories and feeding strategies [56,61] and the overall complexity of the biological and physiological factors which are known to influence the stable isotope ecology of organisms in general [112,113,116–118].

5. Conclusions

Parasite-host trophic fractionation patterns for both ^{13}C and ^{15}N were significantly different when comparing the two different types of parasites (i.e., copepods vs. monogeneans) collected from the

same host species as well as when comparing the same species of parasites (*C. atromaculatus*) collected from the two different species of hosts (*A. coeruleus* vs. *A. bahianus).* Identifying the driving forces behind the wide array of isotope discrimination factors for parasites is a logical next step but requires significantly more scientific attention on the differences in the physiology and feeding ecology of these parasites and their hosts. Furthermore, identifying the specific tissues that are being selected by the parasites and examining how their unique metabolic processes influence isotopic patterns would advance our understanding of their stable isotope ecology. It is increasingly apparent that the stable isotope ecology of host-parasite systems does not always conform to conventional notions regarding the behavior of stable isotopes in ecological systems and these findings contribute to a growing realization that parasite stable isotope ecology requires significantly more scientific attention. This is particularly important given the increasing pressure from parasite minded ecologists to restructure the existing food web models in order to accurately account for parasites [15,16,23,24].

Given the high biomass and diversity of herbivorous reef fishes that includes wide variation in diet, habitat association, behavior, and parasite communities, understanding the role of parasites in their trophic ecology will require studies involving multiple species and localities, as well as analysis of multiple host tissues for stable isotope analysis. In addition, while there was no apparent temporal shift in host blood stable isotopes for either of our host fish, future studies would benefit from a more thorough understanding of the temporal feeding habits of hosts as well as species specific discrimination rates for both hosts and parasites. Future isotope studies of parasites would also benefit from species-specific comparisons of the effects of preservation methods. The demonstrated utility of isotope analysis of preserved and archived specimens may also allow for expanding the inclusion of multiple parasite species and hosts. We hope our findings help stimulate such studies.

Supplementary Materials: The following are available online at http://www.mdpi.com/1424-2818/12/11/429/s1, Table S1: Summary of sampling design, Table S2: Pairwise comparisons of model estimated means, Table S3: Model estimated 95% confidence intervals, Figure S1: Distribution of parasite isotope values relative to host blood for *Neobenedenia sp*. collected from the same host fish. All data presented herein are available at https://doi.org/10.5066/P9QE4FW6.

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