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Enhanced understanding of ectoparasite–host trophic linkages on coral reefs through stable isotope analysis

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ABSTRACT

Parasitism, although the most common type of ecological interaction, is usually ignored in food web models and studies of trophic connectivity. Stable isotope analysis is widely used in assessing the flow of energy in ecological communities and thus is a potentially valuable tool in understanding the cryptic trophic relationships mediated by parasites. In an effort to assess the utility of stable isotope analysis in understanding the role of parasites in complex coral-reef trophic systems, we performed stable isotope analysis on three common Caribbean reef fish hosts and two kinds of ectoparasitic isopods: temporarily parasitic gnathiids (*Gnathia marleyi*) and permanently parasitic cymothoids (*Anilocra*). To further track the transfer of fish-derived carbon (energy) from parasites to parasite consumers, gnathiids from host fish were also fed to captive Pederson shrimp (*Ancylomenes pedersoni*) for at least 1 month. Parasitic isopods had $\delta^{13}C$ and $\delta^{15}N$ values similar to their host, comparable with results from the small number of other host–parasite studies that have employed stable isotopes. Adult gnathiids were enriched in ^{15}N and depleted in ^{13}C relative to juvenile gnathiids, providing insights into the potential isotopic fractionation associated with blood-meal assimilation and subsequent metamorphosis. Gnathiid-fed Pedersen shrimp also had $\delta^{13}C$ values consistent with their food source and enriched in ^{15}N as predicted due to trophic fractionation. These results further indicate that stable isotopes can be an effective tool in deciphering cryptic feeding relationships involving parasites and their consumers, and the role of parasites and cleaners in carbon transfer in coral-reef ecosystems specifically.

1. Introduction

Parasitism, whereby smaller organisms derive energy from larger ones (hosts), usually without killing them, is the most common animal lifestyle and thus the most common consumer strategy (Hudson et al., 2006). It is therefore surprising that, with few exceptions (Price et al., 1986), parasites have historically been ignored in both theoretical and empirical analyses of ecological food webs (Morand and Arias-Gonzalez, 1997; Arias-Gonzalez and Morand, 2006; Dunne et al., 2013). Ecologically-minded parasitologists have called attention to this gap (Marcogliese and Cone, 1997; Wood et al., 2007; Byers, 2009; Johnson et al., 2010), drawing comparisons between parasites and "micropredators" (Raffel et al., 2008) and proposing them as the "ultimate missing link" in our understanding of trophic ecology (Lafferty et al., 2008), with the potential to dominate food-web links. In spite of this, fewer than 10 food-web studies within the past two decades have incorporated parasites (Morand and Arias-Gonzalez, 1997; Arias-Gonzalez and Morand, 2006; Lafferty et al., 2006, 2008; Kuris et al., 2008; Amundsen et al., 2009; Johnson et al., 2010; Hatcher and Dunn, 2011; Sato et al., 2012; Dunne et al., 2013).

Our understanding of food-web ecology, host–parasite interactions, and especially the interface between the two in marine ecosystems lags far behind terrestrial and freshwater systems (Hatcher and Dunn, 2011). A major challenge is that current food-web models are insufficient for inclusion of parasites (Petchey et al., 2008; Sukhdeo, 2010). This gap is particularly apparent for coral-reef systems, where the majority of the biodiversity is comprised of parasites (Rohde, 2002). Parasites can have substantial biomass in marine ecosystems (Kuris et al., 2008) and influence food-web linkages by increasing trophic efficiency (Arias-Gonzalez and Morand, 2006), link density, and connectance (Amundsen et al., 2009). By consuming host tissue, they represent a direct means of host carbon transfer. Indirectly, parasites may influence food-web linkages by altering host movement and other behavioural patterns (Huebner and Chadwick, 2012a, 2012b; Sato et al., 2012; Welicky and Sikkel, 2014).

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Two of the most common external parasites of coral-reef fishes are gnathiid and cymothoid isopods. Gnathiids are small (1–3 mm) protelean parasites that feed on fish blood during each of three larval stages, each of which lives in the substratum between feedings. Thus, they are ecologically similar to terrestrial haematophagous arthropods such as ticks and fleas. After the third feeding, they metamorphose into non-feeding adults, where females produce a single brood and then die (Smit and Davies, 2004). Gnathiid larvae represent a major component of the diet of cleaner fishes, and they are also eaten by cleaner shrimps (Grutter, 1996; Arnal et al., 2000), who remove the parasites from fish hosts. High gnathiid activity even appears to influence the tendency of host fish to visit cleaners (Grutter, 2001; Sikkel et al., 2004). During free-living stages, gnathiids may also be consumed by other microcarnivores (Alldredge and King, 1977; Holzman et al., 2005; Motro et al., 2005), including corals (Artim and Sikkel, 2013). Thus, their potential contribution to food webs is self-evident. Because gnathiids feed during and grow between each of their three larval stages, there is an ontogenetic shift in size with each stage and with each fish host. Consequently, as they grow, their potential role in carbon transport increases because bigger gnathiids can consume, and require, more blood to grow and metamorphose than smaller ones.

Cymothoid isopods of the genus *Anilocra* attach to hosts as juveniles, feed on blood and possibly mucus, epithelium, and subcutaneous tissue (Bunkley-Williams and Williams, 1998). In contrast to gnathiids, *Anilocra* reach large body sizes (up to 25% of host body size – Smit et al., 2014) and remain attached to the host for their entire life, unless they are dislodged or eaten (Ostlund-Nilsson et al., 2005). *Anilocra* females feed on host blood, and likely produce multiple broods during a life span of 12–14 months (Adlard and Lester, 1995). Cleaner species are known to dislodge and consume juvenile cymothoid isopods, which can also be consumed by microcarnivores during free-living stages, but predation on adults is unknown (Bunkley-Williams and Williams, 1998; Ostlund-Nilsson et al., 2005).

Ectoparasites such as these offer a promising starting point for the integration of parasites into food webs. Unlike internal parasites, ectoparasites can often be seen with the naked eye and collected without sacrificing the host. Perhaps most importantly, they can impact food webs through direct consumption by other organisms (Johnson et al., 2010). Stable isotope analysis can provide an indirect measure of parasite trophic ecology, providing an advanced tool in the analysis of host–parasite food-webs (Gómez-Díaz and González-Solís, 2010). Stable carbon isotopes ($\delta^{13}C$) are used to discern carbon or food sources and indicate relative contributions of primary sources to local food webs, with a typical trophic shift of 0–1‰ (DeNiro and Epstein, 1981; McCutchan et al., 2003). In contrast, stable nitrogen isotope ($\delta^{15}N$) values increase with trophic level, typically 2–3‰ (Minagawa and Wada, 1984; Post, 2002; McCutchan et al., 2003), and are useful when estimating trophic level (Post, 2002; McCutchan et al., 2003). Smaller trophic shifts in $\delta^{15}N$ are associated with animals raised on invertebrate diets (1.4 ± 0.2‰, McCutchan et al., 2003). Thus, stable isotope analysis could assist with understanding the complexity of the cryptic trophic relationships involving parasites and other symbionts in biologically complex systems, such as coral reefs.

If parasites function as predators, we would predict a stepwise enrichment in ^{15}N of parasites relative to hosts, on the order of 2–3‰ (DeNiro and Epstein, 1981; Post, 2002). However, applications of stable isotope analysis and published trophic fractionation values to examine parasite–host isotopic relationships have yielded variable results (Lafferty et al., 2008; Doi et al., 2010; Gómez-Díaz and González-Solís, 2010). Isotope patterns are influenced by the feeding strategy or life history stage of the parasite (Iken et al., 2001; Pinnegar et al., 2001; O'Grady and Dearing, 2006), and the level of enrichment can vary in a parasite species found among multiple hosts (Deudero et al., 2002) or among different parasite taxa within hosts (Boag et al., 1997; Neilson et al., 2005; Gómez-Díaz and González-Solís, 2010). In addition, interpretation of results from parasite isotope studies is often limited by the selection of tissue analysed for isotopic comparison with parasites (Power and Klein, 2004; Stapp and Salkeld, 2009). For haematophagous parasites (e.g., gnathiids and *Anilocra*), estimates of trophic shift should be based on isotopic differences between fluids (blood) and consumers (ectoparasites) rather than differences between muscle or bulk tissue and consumers (e.g., McCutchan et al., 2003; Doi et al., 2010), because blood may differ isotopically from muscle tissue or whole organisms (Pinnegar et al., 2001). To date, few studies have examined multiple parasites and hosts simultaneously (Gómez-Díaz and González-Solís, 2010), and no studies, to our knowledge, have incorporated parasite consumers in an isotopic study of food webs.

As a first step in assessing the utility of stable isotope analysis in elucidating the cryptic trophic relationships and carbon transfer mediated by parasites in marine reef systems, we conducted stable carbon and nitrogen isotope analysis of ectoparasitic gnathiid and cymothoid isopods, three fish host species, and one cleaner shrimp species. We hypothesized that host fish, parasite, and parasite consumer will have similar $\delta^{13}C$ values, with little change (0–1‰) in $\delta^{13}C$ with each trophic level. We predicted that parasites would be enriched in ^{15}N by approximately 2–3‰ relative to host heart and blood tissues, due to trophic fractionation of $\delta^{15}N$ (Post, 2002; Stapp and Salkeld, 2009; Schmidt et al., 2011). We calculated the magnitude and direction of fractionation between parasites (gnathiid and *Anilocra*) and host tissues, and between juvenile and adult gnathiid life stages. Lastly, we expected that parasite consumers (cleaner shrimp) will be enriched in ^{15}N relative to gnathiids, consistent with the trophic shift of ~ 1.4‰ reported for invertebrate consumers (McCutchan et al., 2003).

2. Materials and methods

2.1. Study site and organisms

The field portion of the study was conducted during May–August of 2009 and 2010. The primary base of operation was the Virgin Islands Environmental Resource Station in Lameshur Bay, St. John, U.S. Virgin Islands (18°19 N, 65°44 W) and all specimens were collected within Lameshur Bay. Fish species used in the study included longfin damselfish (*Stegastes diencaeus*, Pomacentridae), French grunt (*Haemulon flavolineatum*, Haemulidae), and squirrelfish (*Holocentrus adscenscionis*, Holocentridae). These fish species were chosen based on their distinct differences in habitat use and behaviour, as well as their abundance at our study site. Longfin damselfish are diurnal herbivores that defend year-round territories in shallow reef habitat (Robertson, 1984). French grunts live in shoals or individually in reef habitat during the day and typically migrate to seagrass beds at night where they feed on benthic and demersal invertebrates (Nagelkerken et al., 2008; Appeldoorn et al., 2009). Like French grunt, squirrelfish also refuge in reef structure during the day and are active at night, feeding on demersal and benthic invertebrates and even small fishes. However, in contrast to French grunt, they remain on the reef at night (Randall, 1967; Gladfelter and Johnson, 1983; Menard et al., 2008). Thus, all three fish species demonstrate high site fidelity to resting sites (Nagelkerken et al., 2008) and French grunt in particular may play a significant role in the transfer of parasites among habitats. All three species are infected by the gnathiid isopod *Gnathia marleyi* (Farquharson et al., 2012) at our study sites, although their susceptibility varies (Coile and Sikkel, 2013). In addition, French grunt are infected by the cymothoid isopod, *Anilocra haemuli*, and squirrelfish by *A. holocentric* (Bunkley-Williams and Williams, 1981; Bunkley-Williams, 1984;

Welicky and Sikkel, 2014). At least two of the species, French grunt and longfin damselfish, visit Pederson shrimp (*Ancylomenes pedersoni*) cleaning stations (Sikkel et al., 2004; Huebner and Chadwick, 2012a).

2.2. Sample collection and preparation

The protocol for collection of host fishes and gnathiids was similar to previous studies on gnathiids at this site (Sikkel et al., 2006, 2009; Coile and Sikkel, 2013). Fishes (French grunts and longfin damselfish) were caught from Lameshur Bay on the south side of St. John at night by scuba divers using aquarium nets or in the day by snorkelers using modified cast nets. All divers had extensive experience with collection of live fishes and all fish were handled carefully to minimize abrasion and avoid injury to the fish. After collection, fish were held in a plastic mesh holding cage, and later transferred to plastic containers filled with seawater on a boat or dock for transport to the laboratory. Squirrelfish were not used to collect gnathiids due to their low susceptibility to them. Fish were temporarily (6–30 h) held in a shaded, 1500 L tank with running seawater before being deployed on the reef. Plastic tubes were added to the tank to provide shelter and reduce stress.

Fish were deployed on the reef at dusk by snorkelers. Prior to deployment, each fish was carefully transferred to an individual plastic mesh cage that was placed in a bucket of seawater for transport. On site, cages were placed inside larger cages made of plastic lattice to prevent predation by sharks, and anchored to the substratum with dive weights. The cages were retrieved by snorkelers at times of peak gnathiid activity: dawn and midnight (Sikkel et al., 2006). During retrieval, the cage containing the fish was carefully removed from the protective lattice and swam slowly to the surface to minimize loss of gnathiids. Fish were then placed into a tub of seawater (supplied with an aerating device to ensure adequate oxygen) for 3–4 h to allow attached gnathiids to feed and dislodge from hosts. Prior to removal from the tub, fish were thoroughly rinsed with seawater to dislodge any remaining gnathiids. The water was then strained through a 100 μm plankton mesh. Gnathiids were sorted into three juvenile development stages: P1, P2, and P3, using a dissecting scope, and either frozen for the shrimp feeding experiment described below (P3s), or preserved in 70% ethanol for stable isotope analysis. Gnathiids are extremely fragile, often losing appendages if not preserved immediately. Therefore, to help ensure the integrity of the specimens, gnathiids were preserved for subsequent isotope analysis.

To obtain *Anilocra*, infected fishes (French grunts and squirrelfish) were collected as above then transferred to tanks with running seawater in the laboratory until being processed. *Anilocra* were removed from 10 squirrelfish and six French grunt using forceps. In addition, brooding manca (larvae) were removed from two of the *Anilocra* females collected from *H. adscensionis* ($n = 2$) and *H. flavolineatum* ($n = 2$).

Fish with high gnathiid loads (at least 20) and/or *Anilocra* were sacrificed with an overdose of clove oil (Anderson et al., 1997; Munday and Wilson, 1997; Nickum et al., 2004). Blood was removed from the severed caudal artery of French Grunts ($n = 17$) by capillary tube. The heart and a section of muscle from the rear dorsum were also removed from all fish collected. Heart was used as a proxy for fish blood when it was not possible to remove enough blood from fish for isotope analysis (see below). Abdominal tissue from *Anilocra*, whole gnathiids, and all fish tissues were either placed in 70% ethanol (gnathiids) or frozen (heart, muscle, blood, *Anilocra*) in Whirl Paks at −20 °C.

Gnathiids retrieved from individual hosts were used either for host–gnathiid comparisons or for the shrimp feeding experiment (see below), and less than 1/3 of the total gnathiids were P3 (which, if allowed to metamorphose, turn into adult males or females). This limited the number of adult male and female gnathiids available for stable isotope analysis.

2.3. Gnathiid–shrimp feeding experiment

In order to evaluate the trophic transfer of carbon from fish host to gnathiid parasite to cleaner shrimp, Pedersen cleaner shrimp were collected from their anemone hosts by divers using hand nets, and placed in individual containers within seawater tables. The shrimp were fed ad libitum gnathiids (P3) that were collected from either longfin damselfish or from French grunts. Each shrimp consumed only gnathiids from one species of fish. Throughout the experiment, shrimp were active feeders and consumption of the gnathiid meal was easily confirmed because the blood-engorged gnathiid could be observed in the transparent abdomen of the shrimp. The feeding trial duration was 1.5 months, although some shrimp died before that date. Only shrimp that had fed for 1 month or longer ($n = 8$ for French grunts and $n = 9$ for longfin damselfish) were included in the analysis. The shrimp were preserved in 70% ethanol for stable isotope analysis. Additional Pederson shrimp ($n = 14$) were collected from the same sites within Lameshur Bay and frozen for stable isotope analysis of wild-caught individuals.

2.4. Processing of samples and stable isotope analysis

Fish, shrimp, and *Anilocra* tissues were rinsed with deionized water, dried for 1–2 days at 60 °C, ground, and weighed in tin boats. Gnathiids were rinsed in deionized water, placed directly into preweighed tin boats, and left to dry in the oven over night (60 °C). Because of their small size, multiple individuals of the P1 and P2 gnathiids (3–13 individuals) all from the same host individual were pooled to obtain sufficient sample size for analysis. After drying and cooling, tin boats containing gnathiids and shrimp tissues were acidified with 1% $PtCl_2$ to remove inorganic carbon (Levin and Mendoza, 2007; Demopoulos et al., 2010). Samples were analysed for C and N compositions referenced to Vienna PeeDee Belemnite (VPDB) and nitrogen gas (atmospheric) (Peterson and Fry, 1987). Analyses were performed using a Costech (Valencia, CA, USA) elemental analyzer interfaced to a GV instruments (Manchester, UK) Isoprime isotope ratio mass spectrometer. Reproducibility was monitored using organic reference standards (Fry, 2007) and was 0.05‰ for $\delta^{13}C$ and $\delta^{15}N$. Isotope ratios are expressed as δ values, in units of per mil (‰), where $\delta^{13}C$ or $\delta^{15}N = [(R_{sample}/R_{standard})\text{-}1] \times 10^3$ and R is the corresponding ratio, $^{13}C/^{12}C$ or $^{15}N/^{14}N$.

2.5. Assessment of potential preservation artefact (ethanol vs. freezing)

Studies have reported potential fractionation of ^{13}C and ^{15}N associated with ethanol preservation but indicate that the difference may be species specific (Sweeting et al., 2004; Kelly et al., 2006; Fleming et al., 2011). In particular, for arthropods, including crustaceans, preserved in 70% or 95% ethanol, the resulting fractionation compared with freezing samples has been variable, with either no differences, increases, or decreases in $\delta^{13}C$ and/or $\delta^{15}N$ (Ponsard and Amlou, 1999; Sarakinos et al., 2002; Feuchtmayr and Grey, 2003; Fleming et al., 2011; Krab et al., 2012; Chouvelon et al., 2014). In certain cases, fractionation may have been a consequence of not rinsing off the ethanol prior to drying the tissue and subsequent isotope analysis (Fleming et al., 2011; Krab et al., 2012). In order to estimate whether ethanol preservation potentially influenced the isotopic composition of the gnathiid and shrimp tissue measurements, we compared isotope values from Pederson shrimp and gnathiids frozen and preserved in 70% ethanol. Because of their small size, it was not possible to split these animals in half, preserve half and freeze the other half. This process would have not yielded

sufficient mass for the isotope analysis. However, all the gnathiids were collected from the same fish host individual and all Pedersen shrimp were collected from the same area within Lamershur Bay, USVI. All specimens, whether frozen or preserved in ethanol were first rinsed in DI water, then dried. The dried shrimp were then ground and subsampled into tin boats. Whole gnathiids (P3) were rinsed in DI then dried in tin boats. All samples were acidified using 1% $PtCl_2$ to remove inorganic carbon (Levin and Mendoza, 2007; Demopoulos et al., 2010). Tissue preservation in ethanol had no significant alteration of stable $\delta^{13}C$ (Mann–Whitney U: $U = 28$, $p = 0.274$) or $\delta^{15}N$ (MW: $U = 23$, $p = 0.130$) values for shrimp or gnathiids, MW: $\delta^{13}C$: $U = 2.0$, $p = 0.275$, $\delta^{15}N = U = 1.0$, $p = 0.127$, therefore no correction factor was applied to the stable isotope data.

The carbon and nitrogen isotope discrimination ($\Delta^{13}C$ and $\Delta^{15}N$) between host fish tissues and parasites (P1, P2, P3, adult gnathiids, *Anilocra*) and between adult gnathiids (male and female) and P3 gnathiids were calculated as follows:

$\Delta^{13}C$ and $\Delta^{15}N = (\delta^{13}C$ or $\delta^{15}N$ of parasite$) - (\delta^{13}C$ or $\delta^{15}N$ of host tissue). $\Delta^{13}C$ and $\Delta^{15}N = (\delta^{13}C$ or $\delta^{15}N$ of adult gnathiid-male or female$) - (\delta^{13}C$ or $\delta^{15}N$ of P3 gnathiid$)$.

2.6. Statistical analysis

All data were tested for normality and heteroscedasticity using Shapiro–Wilk and Levene's test for homogeneity of variances (Sokal and Rohlf, 1995). One-way ANOVA was used to test for isotopic differences among French grunt blood, heart, and muscle tissues, using Bonferroni post hoc comparisons. Results from the ANOVA test further allowed us to determine whether heart could be used as a proxy for blood when blood collections were not feasible. Non-parametric Kruskal–Wallis (KW) test was used to test for isotopic differences among longfin damselfish and French grunt tissues and associated gnathiids, and *Anilocra* vs. French grunt and squirrelfish tissues. Significant KW results were followed by post-hoc Mann–Whitney paired comparisons using Bonferroni adjustment for multiple comparisons. Mann–Whitney U tests were used for paired comparisons between longfin damselfish heart and muscle isotope data and between Pederson shrimp and gnathiid isotope data. A significance level of $p < 0.05$ was used for all statistical analyses.

3. Results

3.1. Isotopic composition of tissues

Gnathiids collected from French grunt had the highest $\delta^{13}C$ values (Fig. 1A) and *Anilocra* isopods had the highest $\delta^{15}N$ values (Table 1). In contrast, longfin damselfish hearts had the lowest $\delta^{13}C$ (-13.5 ± 0.4) and $\delta^{15}N$ (6.0 ± 0.3) values (Table 1, Fig. 1B).

3.1.1. French grunts

There were no significant differences between French grunt blood, heart, and muscle $\delta^{13}C$ values (ANOVA, $F_{2,37} = 0.820$, $p = 0.449$). In contrast, blood and heart were significantly depleted in ^{15}N relative to muscle (ANOVA, $F_{2,37} = 25.095$, $p < 0.001$, Fig. 1), but there were no differences in $\delta^{15}N$ values between blood and heart tissues ($p = 0.706$, Fig. 1). Stable carbon and nitrogen isotope values of French grunt tissues and gnathiids also were not different (KW, $\chi^2 = 3.603$, $p = 0.058$ for $\delta^{13}C$, $\chi^2 = 2.006$, $p = 0.157$ for $\delta^{15}N$). While $\delta^{13}C$ values of French grunt blood were similar to the P3 gnathiids (Fig. 2), P3 were generally enriched in ^{15}N relative to blood. There were no significant differences in $\delta^{13}C$ or $\delta^{15}N$ values among different gnathiid larval stages (P1, P2, P3) from French grunt (KW, $\chi^2 = 1.439$, $p = 0.487$ for $\delta^{13}C$ and $\chi^2 = 4.475$, $p = 0.107$ for $\delta^{15}N$).

Similar patterns in the isotope discrimination factors were observed between gnathiids and French grunt blood across juvenile life stages (Table 2), corresponding to negative $\Delta^{13}C$ values and positive $\Delta^{15}N$ values. However, in general, the opposite pattern was observed for discrimination factors calculated for gnathiids and heart tissue and muscle tissue, with slight enrichment in $\Delta^{13}C$ and depletion in $\Delta^{15}N$ (Table 2). Stable nitrogen isotope values were enriched for both females and males relative to P3 gnathiids (Fig. 2), yielding positive discrimination values (Table 2). However, while females were slightly enriched in ^{13}C relative to P3 ($\Delta^{13}C = 0.1 \pm 0.2$), males were depleted in ^{13}C relative to P3 gnathiids, yielding negative discrimination values ($\Delta^{13}C = -2.1 \pm 0.3$).

Table 1
Stable isotopic values and carbon to nitrogen ratios of fish, ectoparasites, and Pederson shrimp from St. John, USVI. Average values (±1 S.E.) and ranges are given.

	N	$\delta^{13}C$	range $\delta^{13}C$	$\delta^{15}N$	range $\delta^{15}N$	average C/N
Stegastes diencaeus						
Fish muscle	9	−12.4 ± 0.2	(−13.1 to −10.8)	7.4 ± 0.1	(6.8 to 8.3)	3.7
Fish heart	9	−13.3 ± 0.3	(−14.8 to −11.8)	6.0 ± 0.3	(4.8 to 7.7)	4.4
Parasites – *Gnathia marleyi* – P3	8	−13.2 ± 0.2	(−14.1 to −12.1)	6.2 ± 0.4	(4.8 to 7.4)	4.7
Parasites – *Gnathia marleyi* – P2	5	−12.8 ± 0.4	(−13.7 to −11.3)	6.7 ± 0.4	(5.1 to 7.6)	4.9
Parasites – *Gnathia marleyi* – P1	4	−12.7 ± 0.2	(−13.3 to −12.3)	6.9 ± 0.9	(5.0 to 8.5)	4.9
Ancylomenes pedersoni (Fed gnathiids from Damselfish)	9	−11.7 ± 0.2	(−12.4 to −10.8)	7.6 ± 0.3	(6.7 to 9.4)	4.5
Haemulon flavolineatum						
Fish muscle	33	−11.0 ± 0.2	(−14.0 to −9.4)	7.9 ± 0.2	(6.3 to 10.1)	3.5
Fish heart	30	−11.1 ± 0.2	(−13.2 to −9.4)	7.1 ± 0.1	(6.1 to 8.4)	3.8
Fish blood	17	−10.8 ± 0.2	(−11.9 to −8.9)	6.6 ± 0.1	(5.7 to 8.0)	3.9
Parasites – *Gnathia marleyi* – P3	24	−11.2 ± 0.3	(−15.3 to −9.4)	6.7 ± 0.1	(5.7 to 9.3)	4.4
Parasites – *Gnathia marleyi* – P2	12	−10.6 ± 0.2	(−11.9 to −9.4)	7.2 ± 0.2	(5.4 to 8.4)	4.3
Parasites – *Gnathia marleyi* – P1	7	−10.6 ± 0.3	(−11.3 to −9.3)	6.8 ± 0.4	(5.5 to 8.7)	4.4
Parasites – *Gnathia marleyi* – adult-female	4	−12.1 ± 0.8	(−14.3 to −11.1)	7.9 ± 0.4	(6.8 to 8.8)	5.3
Parasites – *Gnathia marleyi* – adult-male	4	−14.0 ± 0.8	(−16.4 to −12.7)	7.2 ± 0.3	(6.8 to 8.2)	5.2
Parasites – *Anilocra haemuli* adult female	6	−11.1 ± 0.4	(−12.2 to −9.5)	8.8 ± 0.6	(8.3 to 10.7)	5.6
Parasites – *Anilocra* brood	2	−11.7 ± 1.0	(−12.8 to −10.7)	8.7 ± 1.1	(7.6 to 9.8)	7.0
Ancylomenes pedersoni (Fed gnathiids from Grunts)	8	−11.2 ± 0.1	(−11.8 to −11.0)	7.6 ± 0.3	(6.6 to 9.0)	4.2
Holocentrus adscensionis						
Fish muscle	10	−11.6 ± 0.1	(−12.2 to −11.1)	7.9 ± 0.1	(7.4 to 8.4)	3.7
Fish heart	10	−11.3 ± 0.2	(−12.2 to −10.3)	8.1 ± 0.2	(6.1 to 8.7)	3.6
Parasites – *Anilocra holocentri* adult female	10	−10.9 ± 0.3	(−12.5 to −9.4)	7.7 ± 0.5	(5.2 to 10.2)	5.3
Parasites – *Anilocra* brood	2	−13.1 ± 0.3	(−13.5 to −12.8)	7.8 ± 0.1	(7.8 to 7.9)	7.9
Ancylomenes pedersoni – Wild caught	14	−11.2 ± 0.2	(−12.9 to −10.7)	7.8 ± 0.3	(4.6 to 8.6)	3.4

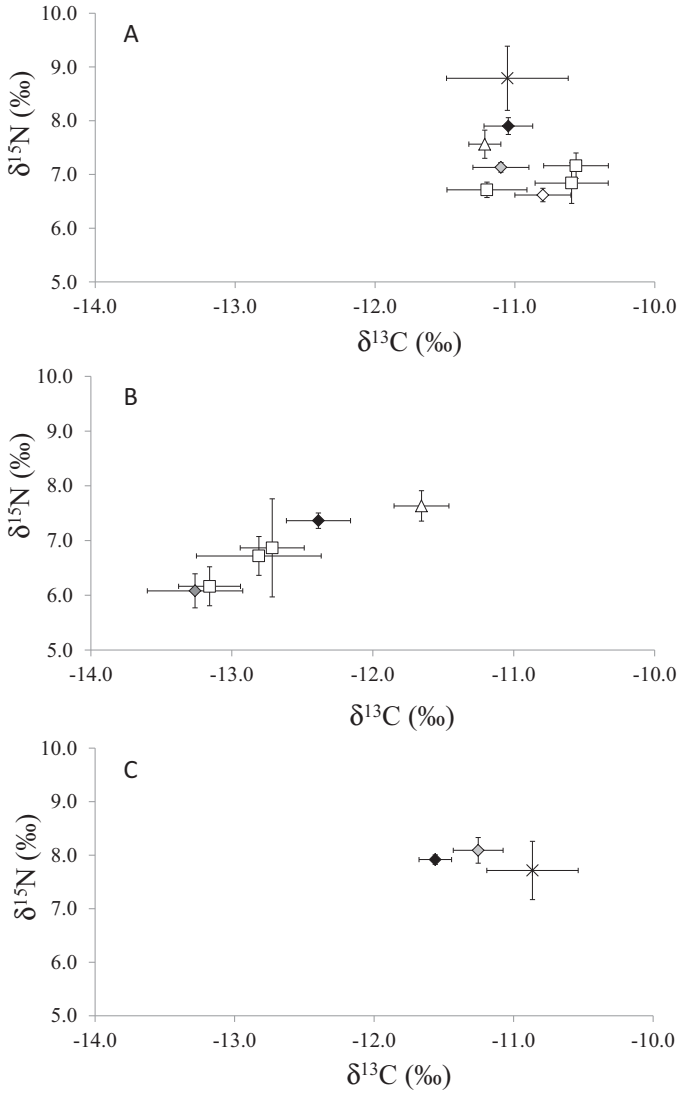

Fig. 1. Mean $\delta^{13}C$ and $\delta^{15}N$ (±1 Standard Error) values of gnathiids (open squares), (A) *Haemulon flavolineatum* heart (grey diamond), blood (white diamond), and muscle (black diamond), Pederson shrimp (*Ancylomenes pedersoni*, open triangle), and *Anilocra* isopods (cross), (B) *Stegastes diencaeus* heart (grey diamond), and muscle (black diamond), and Pederson shrimp (open triangle), and (c) *Holocentrus adscenscionis* heart (grey diamond), and muscle (black diamond), and *Anilocra* isopods (cross).

Anilocra attached to French grunt had similar $\delta^{13}C$ values as their host fish heart and muscle tissues (KW, $\chi^2 = 4.738$, $p = 0.094$). Fish tissue $\delta^{15}N$ values were significantly different from *Anilocra* (KW, $\chi^2 = 8.229$, $p = 0.016$). Post hoc comparisons indicated that while there was no significant difference between *Anilocra* and French grunt muscle $\delta^{15}N$ isotopes (MW: $U = 7.000$, $p = 0.094$), *Anilocra* were significantly enriched in ^{15}N relative to the heart (MW: $U = 5.000$, $p = 0.041$) (Fig. 1A). *Anilocra* broods had similar stable isotope values as their maternal host (Table 1); however, the small sample size ($n = 2$) precluded statistical comparisons. *Anilocra* were significantly enriched in ^{15}N relative to the gnathiids (MW: $U = 12$, $p = 0.008$), but not significantly different in $\delta^{13}C$ (MW: $U = 36$, $p = 0.519$). These two parasite species did not co-occur on the fish analysed in this study. However, the fish were collected in the same location, and there was no significant difference in heart values from the French grunts infested with gnathiids compared with those infested with *Anilocra* (MW: $U = 21.0$, $p = 0.529$ for $\delta^{13}C$, $U = 23.0$, $p = 0.689$ for $\delta^{15}N$).

3.1.2. *Longfin damselfish*

There were no significant differences in $\delta^{13}C$ or $\delta^{15}N$ values among different gnathiid sizes (P1, P2, P3) collected from longfin damselfish (KW, $\chi^2 = 1.380$, $p = 0.501$ for $\delta^{13}C$, $\chi^2 = 1.349$, $p = 0.509$, for $\delta^{15}N$) Thus, isotope values from gnathiids of different juvenile stages were pooled for statistical comparisons with fish tissues (heart and muscle), and cleaner shrimp.

While overall $\delta^{13}C$ values of heart were lower than muscle (Fig. 1B), they were not significantly different (MW: $U = 22.5$, $p = 0.113$). Stable carbon isotope values of longfin damselfish heart, muscle and gnathiids were not significantly different (KW, $\chi^2 = 4.911$, $p = 0.086$), but tissue $\delta^{15}N$ values were significantly different (KW, $\chi^2 = 7.521$, $p = 0.008$). Specifically, longfin damselfish muscle was enriched in ^{15}N relative to heart (MW: $U = 9.000$, $p = 0.004$, Fig. 1B). Stable carbon isotope discrimination factors calculated for gnathiids and heart were variable and slightly depleted for P2 and P3 juveniles, and enriched for $\Delta^{15}N$. Discrimination factors calculated for muscle and gnathiids were similar to heart values for $\Delta^{13}C$, but all $\Delta^{15}N$ values were negative (Table 2).

3.1.3. *Squirrelfish*

In contrast to the French grunt, $\delta^{13}C$ and $\delta^{15}N$ values for squirrelfish heart, muscle, and associated *Anilocra* tissues were not significantly different (Fig. 1C, KW, $\chi^2 = 3.962$, $p = 0.138$ for $\delta^{13}C$, $\chi^2 = 2.429$, $p = 0.297$, for $\delta^{15}N$ respectively). The two *Anilocra* broods analysed were depleted in ^{13}C relative to the adult female, but $\delta^{15}N$ values were similar to their maternal host (Table 1). *Anilocra* discrimination factors were positive for $\Delta^{13}C$ and negative for $\Delta^{15}N$ relative to heart and muscle. Discrimination factors calculated for brood vs. female *Anilocra* were negative for both $\Delta^{15}N$ and $\Delta^{13}C$ (Table 2).

3.1.4. *Shrimp and parasite (gnathiid) feeding experiment*

Shrimp had significantly higher $\delta^{13}C$ and $\delta^{15}N$ values relative to longfin damselfish-fed gnathiids (Fig. 1B, MW: $U = 3.0$, $p = 0.001$ for $\delta^{13}C$, $U = 10.5$, $p = 0.011$ for $\delta^{15}N$). On average, shrimps were 1.3 ± 0.2‰ (range: 0.49–2.15‰) enriched in ^{13}C (similar for predicted trophic fractionation from prey to predator, 0–1‰, DeNiro and Epstein, 1978) and ~1.4 ± 0.3‰ (0.46–3.15‰) enriched in ^{15}N relative to the gnathiids, consistent with trophic shift predicted for consumers of invertebrates (1.4 ± 0.21‰, McCutchan et al., 2003).

There were no significant differences between $\delta^{13}C$ of French grunt-fed gnathiids and shrimp (Fig. 1A, MW: $U = 102$, $p = 0.08$). However, shrimps had significantly higher $\delta^{15}N$ values (7.6 ± 0.7‰) than gnathiids (6.7 ± 0.7‰, MW: $U = 71$, $p = 0.009$), consistent with results from longfin damselfish-fed gnathiids and shrimp consumers. Results indicate that stable carbon isotopes were similar and conserved from host fish, regardless of tissue type (heart, muscle), to parasite, and to parasite consumer. Wild-caught Pederson shrimp $\delta^{13}C$ and $\delta^{15}N$ values were within the range reported for the experiment (Table 1, $\delta^{13}C = -11.2 \pm 0.2$, $\delta^{15}N = 7.8 \pm 0.3$).

4. Discussion

4.1. *Isotopic relationships between parasitic isopods and their fish hosts*

The present study represents the first isotopic analysis of coral-reef parasitic gnathiids and *Anilocra* isopods associated with multiple fish hosts, providing insight into trophic transfer of carbon from host fish to parasite. This is surprising given that *Anilocra* are the most conspicuous ectoparasites of reef fishes and that gnathiids represent the most common reef-based ectoparasites. To date, there have been only two published studies that have applied the stable isotope method to parasitic isopods: *Anilocra physodes*, $N = 6$, associated with

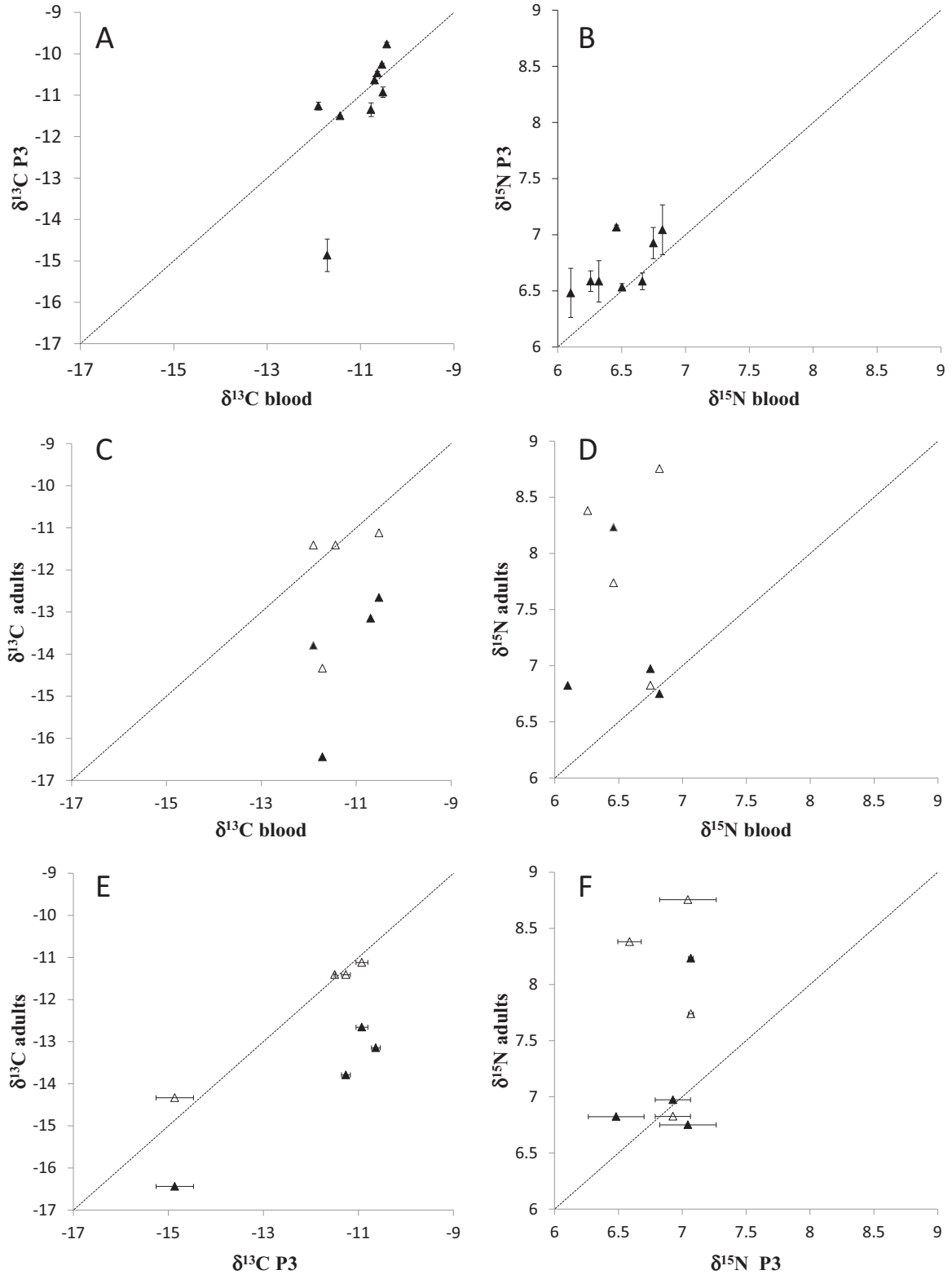

Fig. 2. Stable carbon and nitrogen isotope data for *Haemulon flavolineatum* blood vs. P3 (A and B), blood vs. adult gnathiids (C and D), and P3 vs. adult gnathiids (E and F). Solid triangles represent males and P3s, open triangles represent females. Error bars represent 1 SE of $N = 5$ individual P3 gnathiids analysed from each fish. Dashed line represents 1:1 linear relationship.

seabream (*Boops boops*) (Pinnegar et al., 2001) and deep-sea gnathiids ($N = 16$, no fish host identified or analysed) (Fanelli et al., 2009), but because no fish hosts were identified in the latter study, trophic linkages between parasite and host were not estimated.

Host fish and parasites had similar $\delta^{13}C$ values, consistent with our hypothesis predicting little carbon isotopic change from host-heart and blood to ectoparasite. Neither did we observe an increase in $\delta^{15}N$ from host tissue to juvenile gnathiids, as predicted based

Table 2
Estimates of $\Delta^{13}C$ and $\Delta^{15}N$ discrimination factors (Mean difference ± 1 SE) between host resource and ectoparasites, P3 and adult gnathiids, and anilocra brood and female anilocra.

	Haemulon flavolineatum			*Stegastes diencaeus*			*Holocentrus adscensionis*		
	N	$\Delta^{13}C$ (‰)	$\Delta^{15}N$ (‰)	N	$\Delta^{13}C$ (‰)	$\Delta^{15}N$ (‰)	N	$\Delta^{13}C$ (‰)	$\Delta^{15}N$ (‰)
Gnathia marleyi									
P3-blood	9	–0.3 ± 0.4	0.2 ± 0.1						
P2-blood	7	–0.2 ± 0.2	0.6 ± 0.2						
P1-blood	4	–0.1 ± 0.4	0.2 ± 0.2						
Adult female-P3	4	0.1 ± 0.2	1.0 ± 0.5						
Adult male-P3	4	–2.1 ± 0.3	0.3 ± 0.3						
P3-heart	19	0.1 ± 0.2	–0.4 ± 0.2	9	–0.1 ± 0.4	0.1 ± 0.3			
P2-heart	10	0.1 ± 0.3	–0.2 ± 0.4	6	–0.3 ± 0.4	0.7 ± 0.6			
P1-heart	8	0.4 ± 0.2	–0.3 ± 0.3	4	0.5 ± 0.6	1.4 ± 0.9			
P3-muscle	22	0.1 ± 0.2	–0.7 ± 0.3	12	–0.6 ± 0.2	–1.4 ± 0.3			
P2-muscle	12	0.1 ± 0.4	–0.9 ± 0.4	6	–0.5 ± 0.2	–0.4 ± 0.3			
P1-muscle	8	–0.4 ± 0.2	–1.3 ± 0.4	5	–0.8 ± 0.7	–1.0 ± 0.8			
Anilocra spp.									
Female-heart	6	–0.3 ± 0.4	1.6 ± 0.6				10	0.4 ± 0.3	–0.4 ± 0.5
Female-muscle	6	–1.0 ± 0.5	0.1 ± 0.7				10	0.7 ± 0.3	–0.2 ± 0.6
Anilocra brood-female	2	–0.4 ± 0.4	–0.1 ± 0.5				2	–1.3 ± 0.5	–1.1 ± 0.4

on trophic fractionation. Stable nitrogen isotopes of juvenile gnathiid parasites were depleted relative to muscle, but similar to heart and blood tissue. Given that the heart and blood had very similar isotope values, heart may provide a reliable isotopic proxy for the blood meal consumed by the gnathiids. When gnathiids (P3) were allowed to digest their blood meal, assimilate this source, and metamorphose into adults, there were shifts in both $\delta^{13}C$ and $\delta^{15}N$, for males and females. While there was little change in $\delta^{13}C$ from P3 to females (0.1 ± 0.2), the fractionation from P3 to males was much greater (–2.1 ± 0.3) indicating there might be some differences in how the blood-meal resources are allocated. For females, the blood meal is used for egg development. Lipids are known to be depleted in ^{13}C relative to protein, so it is possible that the decrease in $\delta^{13}C$ from P3 to male may represent preferential incorporation of lipid-rich material. There are major morphological changes that occur when the P3 metamorphoses into a male (e.g., Smit and Davies, 2004, see Graphical Abstract), including the growth of a large head with mandibles. In addition, both % carbon and % nitrogen decreased in P3 (%C = 51, %N = 14) to adult males (%C = 19, %N = 4); thus the isotopic changes may reflect net changes in carbon and nitrogen content in the adult males. Stable isotopic analysis of the molts would assist our understanding of potential carbon and nitrogen losses associated with metamorphosis. There was a slight increase in $\delta^{15}N$ from P3 to males and a greater increase for females (Table 2), which may represent the enrichment in ^{15}N that occurs with trophic fractionation (McCutchan et al., 2003; Stapp and Salkeld, 2009). Additionally, once P3s metamorphose to adults, they no longer feed and the resulting increased $\delta^{15}N$ values may be associated with starvation (e.g., Olive et al., 2003). Increased $\delta^{15}N$ observed here in metamorphosed gnathiids is consistent with what has been observed for terrestrial parasites that have assimilated their blood meal (Rasgon, 2008; Stapp and Salkeld, 2009; Schmidt et al., 2011). However, this is a limited data set ($N = 8$ total for males and females) and provides preliminary insight into isotopic changes associated with assimilation of the blood meal and potential fractionation associated with metamorphosis. Given the small sample size for adult gnathiids reported here, additional work is needed to clarify the isotope fractionation associated with metamorphosis for each life stage. Also, additional fractionation may occur as the metamorphosed parasites age (e.g., Schmidt et al., 2011). Thus, future work should include tracking possible isotopic composition changes as the adults age and prepare to reproduce.

Heart and blood proved to be better isotopic matches to parasites than muscle for $\delta^{15}N$. Results were consistent with published $\delta^{15}N$ data from endoparasites of brook char (*Salvelinus fontinalis*), and threespine stickleback (*Gasterosteus aculeatus*) fishes, and gastropods (*Lymnaea* spp.), which were depleted in ^{15}N relative to host tissues (Power and Klein, 2004; Doi et al., 2010). Differences between gnathiids and fish muscle $\delta^{15}N$ may be due to selective feeding by the gnathiids, including differential selection of isotopically depleted amino acids (Hare et al., 1991). Increases in $\delta^{15}N$ due to trophic fractionation are attributed to a branch point in amino acid metabolism where ^{15}N-depleted ammonia from deamination is lost as urea and ^{15}N-enriched amino acids are incorporated into animal proteins (Gannes et al., 1998). Parasites evolved flexibility in operation of their metabolic pathways, possibly incorporating amino acids directly (O'Grady and Dearing, 2006), rather than synthesizing amino acids de novo (transamination) (Howell, 1976). Also, parasites may minimize transamination and deamination pathways when excess amino acids are present (Kohler and Voight, 1988), resulting in little to no change in $\delta^{15}N$ from host to parasite (Macko et al., 1986). Given that analysed gnathiid juveniles were engorged with blood, it is possible that the blood meal experienced minimal digestion, resulting in negligible fractionation in ^{15}N of the fresh meal relative to host blood.

Isotopic differences between *Anilocra* attached to French grunt vs. squirrelfish may have been a function of the life history of the parasite. *Anilocra* stable isotope values were not significantly different from their squirrelfish host tissues. Results were consistent with published results from *Anilocra* and copepod ectoparasites, where these ectoparasites were not significantly different from their fish host in $\delta^{15}N$ (Pinnegar et al., 2001). In addition, minimal fractionation in ^{15}N was reported between parasitic nematodes living on lizards (O'Grady and Dearing, 2006), rabbits (Boag et al., 1997), and fish (Iken et al., 2001; Pinnegar et al., 2001; Deudero et al., 2002). In contrast, *Anilocra* attached to *H. flavolineatum* were enriched in ^{15}N relative to muscle (0.9‰) and heart (1.7‰, Table 1), although this was lower than previously documented trophic-level fractionation (e.g., 2–3‰, Minagawa and Wada, 1984). Isotopic enrichment in ^{15}N observed in *Anilocra* may be a consequence of multiple factors. *Anilocra* do not feed while brooding, which may result in corresponding isotope differences between brooding and non-brooding isopods. *Anilocra* are known to feed on blood, but they may also feed on other resources, including fish mucus, which may be isotopically distinct from blood and the isotopic composition of the host. In addition, based on published studies of *Anilocra* from the Great Barrier Reef, Australia, females feed on host blood at intermittent times (Adlard and Lester, 1995) and can remain attached to the fish for up to 14 months. Given the periodicity in their feeding on host blood and long periods of attachment, *Anilocra* tissue may not reflect

the same isotope signal as fish heart or blood at the time of collection. Assimilated diet approximated from stable isotope analysis of *Anilocra* could reflect a different time scale than the period recorded in the fish heart tissue. *Anilocra* may have been between feeding periods, resulting in starvation and increased ^{15}N relative to host tissues (Olive et al., 2003). Isotopic enrichment of ^{15}N in parasites relative to hosts from terrestrial and aquatic environments has been reported in multiple studies (Boag et al., 1997; Stapp and Salkeld, 2009; Gómez-Díaz and González-Solís, 2010), indicating that different trophic relationships may exist depending on host and parasite and assimilated food resource.

Significant differences between *Anilocra* and gnathiid $\delta^{15}N$ values attached to French grunt could be due to several factors. One explanation is that the two different parasites are feeding on sources that differ isotopically (e.g., fish blood versus mucus). While *Anilocra* and gnathiids were harvested from different individual fish, there were no isotopic differences among the individual fish that might explain the differences observed in the ectoparasites. Both *Anilocra*-infected fish and fish used to obtain gnathiids were of the same size class and were collected from the same sites and daytime resting shoals and thus likely consumed similar food sources and were exposed to the same environmental conditions. Due to the small organism size, whole gnathiids were required for stable isotope analysis, while dissected abdominal tissue was used for *Anilocra*. Taxon-specific fractionation factors or tissue-specific isotopic differences may have yielded $\delta^{15}N$ differences between these two parasite species. However, results are consistent with studies of both terrestrial and marine parasites that have documented isotopic differences among different parasite taxa within hosts (Boag et al., 1997; Pinnegar and Polunin, 1999; Polunin et al., 2001; Neilson et al., 2005; Gómez-Díaz and González-Solís, 2010).

4.2. Isotopic relationships between host, parasite, and cleaner

To our knowledge, the shrimp feeding experiment presented here also represents the first of its kind, providing insight into trophic transfer of parasite carbon to parasite consumers. Only gnathiids were used for this study and the experiment yielded slightly different results for damselfish than for grunt-fed gnathiids. For damselfish, shrimp were enriched in ^{13}C relative to gnathiids on average 1.3 ± 0.2‰ (range: 0.49–2.15‰). These results may be a function of different lipid content between gnathiids and shrimp. Lipids are more depleted in ^{13}C than proteins or carbohydrates and blood has a high lipid content (Focken and Becker, 1998; Pinnegar and Polunin, 1999). Presence of lipids can affect fractionation because the synthesis of lipid discriminates against the heavier isotope (^{13}C) in favor of the lighter isotope (^{12}C) (DeNiro and Epstein, 1981). Gnathiids may contain high concentrations of lipids due to their blood meal, resulting in depleted ^{13}C values complicating comparisons with whole shrimp isotopic composition. However, the C/N values for shrimp and gnathiids do not indicate that lipids were a significant factor influencing $\delta^{13}C$ values (Table 1, e.g., Post et al., 2007). Another possibility is that the experiment did not extend long enough and the shrimp were unable to equilibrate with the gnathiid food meal. However, it is unlikely that this was the cause of the carbon isotopic differences observed because shrimp were fed over the same time period for both the damselfish and grunt experiments and there were no significant differences in isotopes between grunt-fed gnathiids and shrimp. If the standard 0–1‰ shift associated with trophic fractionation of carbon (DeNiro and Epstein, 1981) is applied to the gnathiid isotope data ranges (–14.1 to –11.3‰, Table 1), the result (–13.1 to –10.3‰) overlaps with the range in $\delta^{13}C$ values of shrimp (–12.4 to –10.8‰). It is probable that the changes in $\delta^{13}C$ values from parasite to consumer reflect typical trophic fractionation of carbon.

Given the importance of cleaning symbioses on coral reefs, it is surprising that stable isotopes have not been applied to decipher these trophic relationships before this study. Most work on cleaning symbioses has focused on cleaner fishes; however, relatively little has been done on cleaner shrimps. In reef environments, 40 species of shrimp and other decapods are considered cleaners of reef-associated fish (Côté, 2000); of these only five species have been shown to remove ectoparasites. Becker and Grutter (2004) found gnathiid isopods and copepods in the gut contents of wild cleaner shrimp, *Urocaridella* sp. and *Periclimenes holthuisi* from the Great Barrier Reef, Australia and captive shrimp ate monogenean flatworms. In the temperate Atlantic, Ostlund-Nilsson et al. (2005) similarly found that two species of shrimps fed on both crustacean and monogenean parasites. Only *A. pedersoni* has been shown to effectively remove parasites in the Caribbean, and is known to eat juvenile *Anilocra* (Bunkley-Williams and Williams, 1998), monogeneans (McCammon et al., 2010), and gnathiids (this study). While ours is the first study of its kind, it examined only one cleaner species, two kinds of parasite (two genera of isopods), from three hosts. Future studies that incorporate additional fish species, parasites (e.g., monogeneans), and cleaners (gobies and wrasses), as well as other consumers of parasites such a microcarnivorous fishes and even corals will enable the development of food-web models that include host–parasite and parasite–consumer trophic dynamics in reef environments.

5. Conclusions

In conclusion, this is the first study to document trophic interactions via stable isotope analysis using multiple hosts, parasites and parasite consumers. In addition, this represents the first study to estimate isotopic fractionation between parasites and host tissues that includes analysis of pre and post-metamorphosis parasite life stages (P3 and adult gnathiids). Blood, heart, and muscle $\delta^{13}C$ values were consistent with parasite values, thus trophic transfer of carbon could be elucidated from analysis of the muscle and the parasite. However, heart and blood $\delta^{15}N$ values were closer to parasites relative to muscle, indicating that heart was a better proxy than muscle for the parasite blood meal. Isotopic similarity in heart, blood, and gnathiid $\delta^{13}C$ and $\delta^{15}N$ values suggests dependency and selectivity of that tissue (i.e., blood). Thus, in order to enhance the success of stable isotope analysis in parasite-food web applications, results indicate a clear need to identify possible food resources to parasites and select that tissue for isotope analysis, whenever possible. Given that gnathiids and *Anilocra* had similar $\delta^{13}C$ values to their fish hosts, $\delta^{13}C$ analysis of ectoparasites may represent a non-lethal method to estimate host carbon source and elucidate trophic dynamics. This information can lead to understanding of habitat use/resource use, and be adopted for ecosystem scale studies by incorporating a broader range of parasite hosts and parasite consumers.

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Conflict of interest

The authors declared that there is no conflict of interest.

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