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Stable Isotopic Evidence of Mixotrophy in Xylophagaids, Deep-Sea Wood-Boring Bivalves

Janet R. Voight[1], Jacob C. Cooper[1,2] and Raymond W. Lee[3]*

[1] Negaunee Integrative Research Center, Field Museum of Natural History, Chicago, IL, United States, [2] Committee on Evolutionary Biology, The University of Chicago, Chicago, IL, United States, [3] School of Biological Sciences, Washington State University, Pullman, WA, United States

Deep-sea wood-boring xylophagaid bivalves are thought to ingest only wood and to use nitrogen fixed by their symbiotic microbes. Reconsidering this assumption, we tested whether $\delta^{13}C$ and $\delta^{15}N$ isotopic values of ten species in four xylophagaid genera collected between 18 m and 4626 m depth suggest that some may use different trophic strategies. Isotopic signatures of six species were entirely consistent with predicted xylophagy, but four species, three members of the *Xylophaga dorsalis* clade and *Abditoconus heterosiphon*, had $\delta^{15}N$ signatures over 3.7‰, significantly higher than the value predicted for nitrogen fixed by bacteria. These species may supplement freshly fixed nitrogen with an alternate source, such as particulate organic material. Although the animals' reduced palps and the lack of microbes in the gut of laboratory-maintained specimens were cited as arguing against filter feeding, the animals may filter feed opportunistically. *A. heterosiphon* was unique in having $\delta^{13}C$ signatures more negative than the wood into which they bored, inconsistent with cellulose consumption, and $\delta^{15}N$ values higher than predicted for nitrogen fixed by bacteria. We suggest that greater trophic diversity may exist among xylophagaids than has been expected. Ecological studies of wood-fall communities cannot assume that the entire community is sustained only by wood-bound energy. We attribute variation in $\delta^{13}C$ signatures among 42 specimens of *X.* s.l. *zierenbergi* from six collections, five at the same depth, to variation in the type of wood bored. Investigators beginning food web studies should sample xylophagaids and wood itself; if the deployment is large, repeated samples of the wood, closely matched to xylophagaids, may be necessarily to fully define the substrate's stable isotopic signal.

Keywords: carbon isotope, nitrogen isotope, *Xylophaga*, *Abditoconus*, *Xyloredo*

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****Correspondence:***
Janet R. Voight
jvoight@fieldmuseum.org

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INTRODUCTION

Wood-boring bivalves of the Xylophagaidae are thought to bore into, ingest and digest sunken wood (Distel and Roberts, 1997); in doing so they are thought to sustain diverse communities associated with wood that has sunken to the deep-sea floor (Turner, 1973). The importance of the bivalves in making the wood's energy and nutrients accessible to other animals has lately been questioned; bacteria may play a larger role than has been assumed (e.g., Kalenitchenko et al., 2018).

This may be symptomatic of how little we know about xylophagaids; the composition of their diet has been poorly documented, but wood is widely accepted to be their main food source.

Anatomy has contributed to the belief that xylophagaids are strict xylophages. Palp size may predict filter feeding ability in teredinids (Saraswathy and Nair, 1971), the sister taxon of the xylophagaids (Distel et al., 2011). *Xylophaga dorsalis* has reduced palps and gills that lack strong sorting mechanisms (Purchon, 1941), features seemingly indicative of reduced filter feeding ability. However, xylophagaids that bored into the gutta-percha cover of transatlantic cables (Web, 1900) would have had to filter feed (Purchon, 1941), as would those that bored into plastics and vinyl (Muraoka, 1967). Those that were recovered from plastics were, however, half the size of those recovered from wood deployed on the same rack at 722 m for 13.4 months at about 5°C (Muraoka, 1967). These data suggest that, while possible, life without wood is less than ideal. Ansel and Nair (1969) contended, without citing supporting data, that like their relatives the boring pholadids, xylophagaids use wood for support and protection rather than for food.

More recent, data-based arguments for the animals' strict reliance on xylophagy include that individuals of *Xylophaga washingtona* that had been held in wood in seawater tanks had few microorganisms among the wood shavings in their gut (Distel and Roberts, 1997). The gills of the two xylophagaid species (*X. washingtona* and *Xylophaga* s.l. *atlantica*) examined supported dense endosymbiotic bacteria, thought to help digest wood as they do in teredinids (Sabbadin et al., 2018). Teredinids appear able to survive by xylophagy, but studies using radio-labeled substrates and $\delta^{13}C$ and $\delta^{15}N$ isotopic analyses find some teredinids, e.g., *Lyrodus pedicellatus*, *Teredo navalis, Bankia carinata*, filter feed at least opportunistically (Pechenik et al., 1979; Gallager et al., 1981; Paalvast and van der Velde, 2013; Charles et al., 2018). Karande et al. (1968), cited by Pechenik et al. (1979) stated that *T. fucifera* required phytoplankton to grow and reproduce.

Of five studies of xylophagaid $\delta^{13}C$ and $\delta^{15}N$ stable isotopic signatures (Nishimoto et al., 2009; Bernardino et al., 2010; Yamanaka et al., 2015; Gaudron et al., 2016; Zapata-Hernández et al., 2016), only Gaudron et al. (2016) postulated xylophagaid filter feeding. Dwarf males of *Xylophaga* s.l. *atlantica* removed from wood that had been deployed at 2279 m depth near hydrothermal vents on the Mid-Atlantic Ridge had enriched $\delta^{13}C$ and $\delta^{15}N$ isotopic values. In this dense population, dwarf males were concluded to filter feed on fecal matter and other by-products of large, autonomously boring females.

In terms of $\delta^{13}C$ isotopic values, strictly xylophagous xylophagaids are predicted to be 1–2‰ enriched, that is more positive, than the wood they ate, and that the fecal chimneys of members of the *X. dorsalis* clade would be depleted, or more negative than wood. Because the bivalves digest cellulose (Purchon, 1941; Nishimoto et al., 2009), their fecal chimneys would be composed mainly of lignin. The $\delta^{13}C$ signature of cellulose is enriched relative to that of lignin (Loader et al., 2003). If they ingested sulfide-oxidizing bacteria, their $\delta^{13}C$ isotopic values may be depleted relative to the wood. Wood, however, is less than ideal for stable isotopic studies. Even within an individual tree, wood can have a surprisingly large range of $\delta^{13}C$ signatures (e.g., Leavitt and Long, 1986; Schleser et al., 2015). Weather, age, heartwood versus sapwood, water stress, even sunlight exposure all reportedly affect wood's $\delta^{13}C$ signature by over 2‰ (e.g., Bert et al., 1997; Korol et al., 1999; Loader et al., 2003; Taylor et al., 2007).

In terms of $\delta^{15}N$ isotopic values, strictly xylophagous xylophagaids are predicted to have mean $\delta^{15}N$ values from −2 to + 3‰. The extremely low nitrogen concentrations in wood, 0.08–0.2% (Filipiak, 2018), force xylophagous animals to get supplemental nitrogen. To do so, xylophagaids are thought to rely on bacteria symbiotic in their gills (Distel and Roberts, 1997). In addition to their physical presence, two bacterial species from the gills of *X. dorsalis* show a 95% genetic similarity to bacteria in the gills of teredinids that are known to fix nitrogen; other bacterial species that are more distantly affiliated with teredinid symbionts occur in other members of *X. dorsalis* (Fagervold et al., 2014). Stable isotopic signatures of nitrogen fixed by bacteria are between −2 and 0‰ (Somes et al., 2010). Ingestion of symbionts or recycling of nitrogen (Ferrier-Pagés and Leal, 2019) could expand the range of $\delta^{15}N$ values in xylophages to −2 to + 3‰, assuming conventional trophic enrichment of 3.4 ± 1.1‰ (Minagawa and Wada, 1984). Values exceeding 3‰ suggest use of a different nitrogen source, such as Particulate Organic Material (POM).

To test whether xylophagaids rely exclusively on xylophagy and nitrogen-fixing bacteria, we apply $\delta^{13}C$ and $\delta^{15}N$ stable isotopic analysis to 10 xylophagaid species from four clades collected from 18 to 4626 m depths. If a species $\delta^{13}C$ value is 1–2‰ enriched relative to the wood it bored and its $\delta^{15}N$ is between −2 and 3‰, it will be viewed as xylotrophic, the null hypothesis. Species that use POM would be revealed by enriched $\delta^{15}N$ values; species with depleted $\delta^{13}C$ values compared to the wood it bored might ingest sulfide-oxidizing bacteria. We also report analyses of six collections of one species. With stable isotopic studies of xylophagaids so rare, and often with no or unreliable species identifications, we hope to provide a framework for future studies of these animals.

MATERIALS AND METHODS

Specimens and Wood Analyzed

Seventy-one specimens from ten localities deposited in Field Museum of Natural History (FMNH) collection and one specimen from TePapa National Museum of New Zealand (NMNZ) collection are the basis of this study (**Table 1**). Of these, 42 are members of *Xylophaga* s.l. *zierenbergi* removed from six deployments. The specimens differed in their source, whether from wood randomly encountered on the seafloor (i.e., "wild" wood), or wood experimentally deployed on or above the sediment and in their preservation histories. Fixation in formalin followed by storage in 70% ethanol or preservation and storage in 95% ethanol were the alternative means of preservation.

We must address two taxonomic issues. First, the genus *Xylophaga* is paraphyletic (Voight et al., 2019). The type species of

TABLE 1 | The taxon, depth, locality, wood type, deployment duration (if applicable), preservation (F, E formalin to ethanol; 95 E 95% ethanol), mean tissue $\delta^{13}C$ value (standard deviation) and number of specimens, mean tissue $\delta^{15}N$ value (standard deviation) and number of specimens, mean tissue C/N ratio (standard deviation), mean wood $\delta^{13}C$ value (standard deviation), mean wood $\delta^{15}N$ value (Standard deviation), wood C/N ratio (standard deviation) and the source of these specimens.

Taxon	Depth (m)	location	Wood type, deployment duration, preservation	Mean tissue $\delta^{13}C$‰ (Std Dev) n	Mean tissue $\delta^{15}N$‰ (Std Dev) n	C/N tissue (Std. Dev)	Wood $\delta^{13}C$ (Std Dev) n	Wood $\delta^{15}N$ (Std Dev) n	Wood C/N (Std Dev)	References
Xylophaga dorsalis Bergen, Norway	210	60º 28.915' N 5º 25.065' E	Wild 95 E	−21.8 (0.03) *n* = 2	6.3 (0.2) *n* = 2	3.7 (0.1)	−28.1 (0.4) *n* = 4	−1.3 (1.6) *n* = 4	28.9 (13.7)	Unpub.
X. washingtona Friday Harbor, WA United States	18	48° 32.732' N 123° 0.78' W	Wild 95 E	−23.2 (1.0) *n* = 4	7.2 (0.35) *n* = 4	4.6 (1.2)	–	–	–	Unpub.
X. oregona* inactive Juan de Fuca Ridge	2211	47° 56.781' N 129° 5.822' W	Fir 24 mo F, E	−22.4 (0.2) *n* = 3	1.5 (1.6) *n* = 3	4.3 (0.03)	−24.3 (0.2) *n* = 3	0.1 (0.9) *n* = 3	863.9 (667)	Voight (2007)
Xylophaga alexisi Cape Verde Abyssal Plain	4626	21° 03.81'N 31° 12.21'W	7 mo F, E	−24.5 (0.6) *n* = 4	4.4 (0.8) *n* = 4	4.3 (0.3)	−26.0 (0.1) *n* = 2	0.2 (0.7) *n* = 2	140.3 (56.6)	Voight and Segonzac (2012)
Xylophaga s.l. *microchira* Cascadia Basin	2639	47° 42.637' N 127° 47.625' W	Fir 10 mo F, E	−23.1 (0.4) *n* = 2	−1.0 (0.6) *n* = 2	3.8 (0.2)	−24.6 (0.1) *n* = 2	0.2 (0.7) *n* = 2	239.8 (158)	Voight (2007)
X. s.l. *microchira* Cascadia Basin	2639	47° 42.637' N 127° 47.625' W	Fir 10 mo F, E	−22.9 (0.28) *n* = 2	0.9 (0.02) *n* = 2	3.8 (0.4)	−24.6 (0.1) *n* = 2	0.2 (0.7) *n* = 2	239.8 (158)	Voight (2007)
Xylophaga s.l. *muraokai** inactive Juan de Fuca Ridge	2211	47° 56.781' N 129° 5.822'W	Fir 24 mo F, E	−23.1 (0.04)*n* = 2	1.3 (0.3) *n* = 2	4.1 (0.1)	−24.3 (0.2) *n* = 3	0.1 (0.9) *n* = 3	863.9 (667)	Voight (2007)
X. s.l. *muraokai* Monterey Canyon	3100	36°15.677'N 122°40.679'W	Ginkgo 24 mo 95 E	−24.1 (0.3) *n* = 3	1.7 (0.7) *n* = 3	4.0 (0.2)	–	–	–	Judge and Barry (2016)
Xylophaga s.l. *zierenbergi** inactive Juan de Fuca Ridge	2211	47° 56.781' N 129° 5.822'W	Fir 24 mo F, E	−23.0 (0.2) *n* = 2	0.3 (0.09) *n* = 2	4.2 (0.2)	−24.3 (0.2) *n* = 3	0.1 (0.9) *n* = 3	863.9 (667)	Voight (2007)
Xylophaga s.l. *zierenbergi* Monterey Canyon	3100	36°15.677' N 122°40.679'W	Various 24 mo	−24.5 (1.5) *n* = 40	−0.1 (1.1) *n* = 40	3.9 (0.29)	–	–	–	Judge and Barry (2016)
Abditoconus heterosiphon Cascadia Basin	2658	47° 45.755' N 127° 45.441' W	Fir 10 mo F, E	−25.3 (0.2) *n* = 4	3.7 (0.3) *n* = 4	5.7 (0.2)	−23.9 (*n* = 1)	1.1 *n* = 1	263.4	Voight (2007)
Xylopholas crooki Monterey Canyon	3,000–3,150	36° 29.962' to 36° 27.973' N 122° 38.313' to 122° 36.7' W	Wild F, E	−25.8 (1.1) *n* = 2	1.5 (0.08) *n* = 2	3.9 (0.2)	–	–	–	Voight (2016)
Xyloredo nooi NMNZ M.084306 New Zealand	517– 518	43° 57.28' S 176° 46.03' E	Wild frozen F, E	−22.4 *n* = 1	0.8 *n* = 1	5.2	–	–		Voight et al. (2019)

*Species in the Xylophaga dorsalis clade in bold. * Indicates specimens of the three species removed from the same piece of wood. Specimens of X. alexisi from wood were suspended at least 50 cm above the sediment; those of X. s.l. zierenbergi from Judge and Barry (2016) are pooled from 5 deployments, including wood of pine, spice bush, oak, ginkgo and island ironwood (**Figure 2**). – indicates not measured.*

the genus, *X. dorsalis*, forms a clade with several species included here (*X. washingtona, X. oregona, X. alexisi*). The other species named in the genus do not share a recent common ancestor with the name-bearing clade. To highlight their independent origin, despite the shared genus name, we apply "s.l." to their names. Second, when citing literature reports, we used the species names assigned in the original paper, except for Zapata-Hernández et al. (2016). They applied the name of the North Atlantic species, *X. dorsalis*, to their Chilean specimens; we use instead the name *X. globosa*, erected for a species from Valparaiso, Chile, about 8° north of the collection site.

Where available, the stable isotopic values of the wood into which the animals bored were determined. Wood's very low nitrogen concentration, roughly 0.1% that in insect bodies (Filipiak and Weiner, 2014), not only forces xylophages to find an alternate nitrogen source, it made accurately determining its $\delta^{15}N$ signature extremely difficult in our laboratory. The nitrogen values we report from wood must be considered with caution. POM from our collection localities are not available. Globally, average deep-sea heterotrophic animals have isotopic values ranging from 10–13‰ for $\delta^{15}N$ and −17 to −21‰ for $\delta^{13}C$, depending on latitude (reviewed in Parzanini et al., 2019). POM data from the literature show elevated values compared with nitrogen fixed by bacteria. Examples are: tropical Atlantic (POM 300 m depth 6–8‰, Montoya et al., 2002), North Sea and Coastal Norway (benthic POM average 6‰, Silberberger et al., 2019), Northeast Pacific (surface POM average 8‰, Altabet et al., 1999).

Specimen Preparation

Wood, bivalves and fecal chimneys were sampled with a sterile scalpel; the samples were air-dried or placed in 95% ethanol then air-dried. Samples (0.3–0.7 mg dry weight) were rinsed in distilled water, dried, then packaged in tin capsules for mass spectrometry, and analyzed using a Costech (Valencia, CA, United States) elemental analyzer interfaced with a continuous flow Micromass Isoprime isotope ratio mass spectrometer (EA-IRMS) for $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratios. Measurements are reported in δ notation [per mil (‰)] and ovalbumin was used as a routine standard. Precision for $\delta^{13}C$ and $\delta^{15}N$ was ± 0.2‰ and ± 0.4‰ (S.D. of 10 replicate standards), respectively.

We analyzed the siphon, gills, mantle and cecum of specimens of *X. dorsalis* individually to test for organ-specific biases. The wood-filled cecum had a $\delta^{13}C$ isotopic value reflecting its contents, being 2‰ depleted compared to the muscle and gills which at −21.7‰ and −22.0‰ were indistinguishable given our precision. Gills had the lowest $\delta^{15}N$ value at 4.3‰, the cecum was intermediate at 5.1‰ and the muscle was 6.1‰. Complete data are reported in **Supplementary Table S1**. We opted to use the siphons for the remaining analyses due to their ease of accessibility and assumed comparability.

Data Analyses

We compared the mean $\delta^{13}C$ isotopic signatures of seven bivalve species to those of subsamples of the wood into which they had bored; wood samples were lacking for the other taxa. For *X. dorsalis* and *X. oregona*, we also compared the $\delta^{13}C$ and $\delta^{15}N$ isotopic signatures of the chimneys to those of tissue and wood. To examine variation with depth, we plotted the mean $\delta^{15}N$ values of each species versus the species' collection depth, pooling the five Monterey Canyon collections of *X.* s.l. *zierenbergi*. We calculated the correlation coefficient in Excel©, both with and without the relatively shallow species.

Small within-species sample sizes limited the ability of statistical tests to assess the significance of differences. We calculated 95% confidence intervals of the mean nitrogen isotopic values of each species in Excel©, after pooling species from multiple sites. Resultant intervals that did not overlap are thus significantly different at $p < 0.05$ (Zar, 1999).

We compared the $\delta^{13}C$ isotopic values and the $\delta^{15}N$ isotopic values of our chemically preserved specimens to isotopic values from literature accounts that were based on frozen tissues, using the rank sum test calculated by hand.

Because multiple variables impact each sample, we performed multiple tests in R 3.6.1 (R Core Team, 2019), as described here. Using clade, depth, $\delta^{13}C$, wood type, taxon, and preservation method, we assessed variable importance for determining $\delta^{15}N$ using Random Forests in the package "ranger" (Wright and Ziegler, 2017), manipulating our data using the packages "data.table" (Dowle and Srinivasan, 2019) and "tidyverse" (Wickham, 2017) with reference to the Random Forest pipeline of Johnston et al. (2019). Data were visualized using "ggplot2" (Wickham, 2016). To assure that phylogeny was not overly biasing our data, we compared the nitrogen isotopic values for species that were included in a phylogenetic analysis (Voight et al., 2019) using a phylogenetically corrected ANOVA in the R package "phytools" (Revell, 2012). The phylogenetic tree was pruned to include only species represented in the isotopic analysis; each species then had one tip randomly selected to provide a phylogenetic distance for the test. Lastly, non-parametric Kruskal-Wallis tests were performed using the R function "kruskal.test" to determine significant differences between different variable combinations. Notably, we used this to assess how chemical preservation affected the stable isotopic signatures independent of species membership. Complete code for the analyses is presented in **Supplementary Information**.

RESULTS

The $\delta^{13}C$ values for the 72 individual xylophagaids analyzed ranged from −26.6 to −21.5‰; their $\delta^{15}N$ values ranged from −2.3 to 7.7‰ (**Figure 1** and **Table 1**). Most individuals and species analyzed had $\delta^{13}C$ values enriched 1.2 to 1.7‰ over the wood into which they bored (**Table 1**); their $\delta^{15}N$ isotopic values were generally between −2 and 2‰ (**Figure 1** and **Table 1**). Only in *Abditoconus heterosiphon* were $\delta^{13}C$ values depleted relative to the wood they bored, being 1.4‰ more negative. Three members of the *X. dorsalis* clade (*X. washingtona*, *X. dorsalis, X. alexisi*) and *A. heterosiphon* had both mean $\delta^{15}N$ values (**Table 1**) and 95% confidence intervals >3.0 (**Table 2**); they thus significantly exceeded the value predicted for freshly fixed nitrogen. The confidence intervals of *X. dorsalis* and *X. washingtona* did not overlap those of any other species (**Table 2**); their $\delta^{15}N$ values are

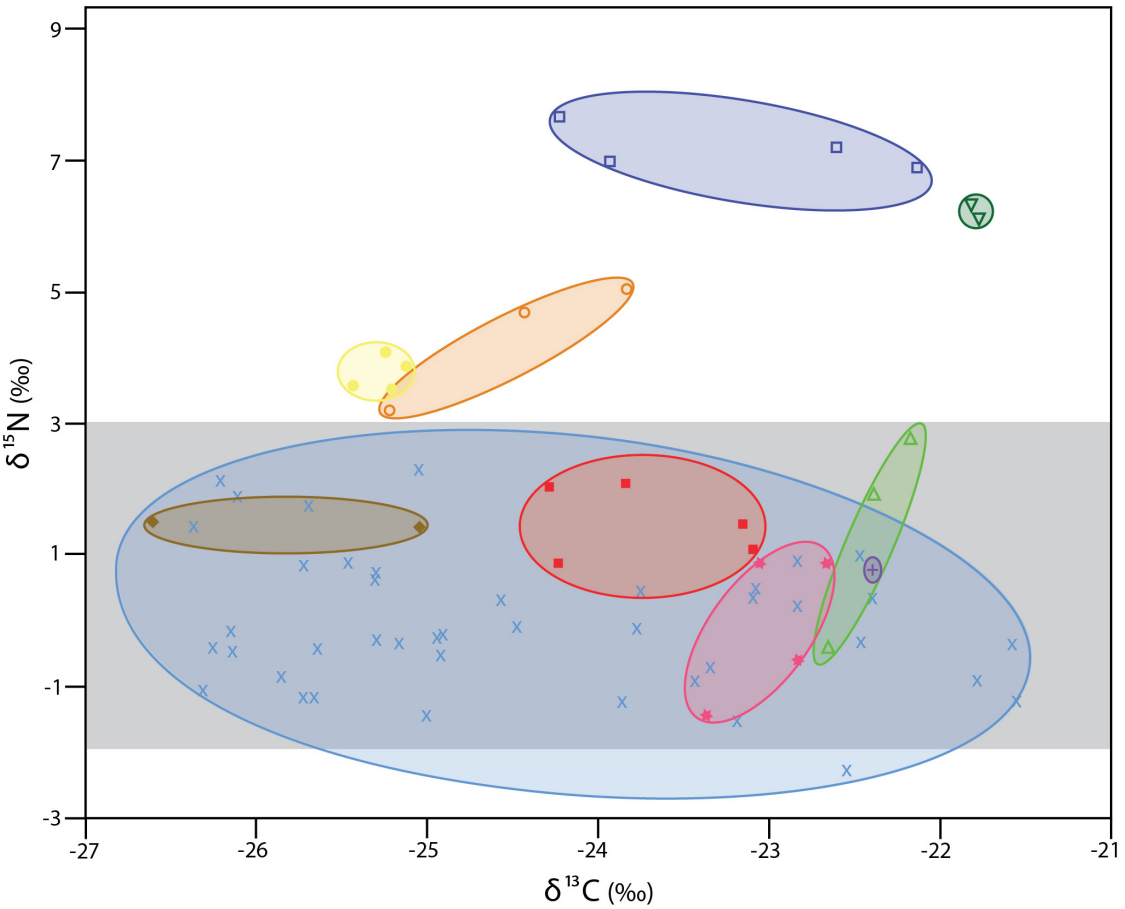

FIGURE 1 | $\delta^{13}C$ and $\delta^{15}N$ values for specimens analyzed here. Ovals unite species. All members of the *X. dorsalis* clade are represented by open symbols. Dark blue open squares *X. washingtona*; dark green open, upside-down triangles *X. dorsalis*; orange open circles *X. alexisi*; green open triangles *X. oregona*. Yellow solid circles *A. heterosiphon*; brown solid diamonds *Xylopholas crooki*; red solid squares *Xylophaga* s.l. *muraokai*; pink solid stars *Xylophaga* s.l. *microchira*; purple plus sign *Xyloredo nooi*; blue crosses *Xylophaga* s.l. *zierenbergi* from six pooled collections (for details see **Table 1**). The area highlighted in light gray is that consistent with nitrogen fixed by bacteria (–2 to + 3).

thus significantly enriched ($p < 0.05$) relative to those of every other taxon we analyzed.

Our ANOVAs failed to find any significant differences with respect to $\delta^{13}C$ and $\delta^{15}N$ after phylogeny was taken into account. Different iterations result in different p values, but we found these values to be ca. $p \approx 0.3$ and $p \approx 0.2$, respectively.

Preservation Artifact?

Differences in isotopic values among species do not appear to be due to preservation. *X. dorsalis* and *X. washingtona* were preserved in 95% ethanol, as were all members of *X.* s.l. *zierenbergi* from Monterey Canyon (**Figure 1**). *Xylophaga alexisi* and *A. heterosiphon*, the other species with elevated $\delta^{15}N$ values, were fixed in formalin and were stored in 70% ethanol, as were the Endeavour specimens of *X.* s.l. *zierenbergi*, *X.* s.l. *muraokai* and *X.* s.l. *microchira* with typically xylophagous $\delta^{15}N$ values (**Figure 1**). The results are also robust to the duration of preservation. The four outstanding species include *X. dorsalis*, collected 1 year before the analysis, and *X. alexisi* collected 27 years before the analysis. These are the extremes among the specimens considered.

Abditoconus heterosiphon had $\delta^{13}C$ values depleted relative to the wood they bored and enriched $\delta^{15}N$ values. The specimens were collected and preserved on the same cruise, using the same chemicals, as were those of *Xylophaga* s.l. *microchira*, which had isotopic signatures entirely consistent with strict xylotrophy (**Tables 1**, **2**). Kruskal-Wallis tests found significant effects of preservation type ($p = 0.005$), but the formalin-fixed, ethanol-stored group contained many individuals of the *X. dorsalis* clade and *A. heterosiphon* (11 of 21) while the ethanol-preserved specimens included a comparative few of these (6 of 51).

The $\delta^{13}C$ values of the 40 specimens of *Xylophaga* s.l. *zierenbergi* removed from different types of wood by Judge and Barry (2016) and likely preserved in the same batch of 95% ethanol exceeded the isotopic $\delta^{13}C$ range of the other 32 specimens considered, regardless of different chemicals used to preserve the latter (**Figure 1**).

TABLE 2 | Reported are the number of samples, the mean δ^{15}N value, 95% confidence interval of the mean (CI) and the lower and upper 95% confidence intervals for each of the nine species represented by more than one specimen treated here; specimens of *X*. s.l. *zierenbergi*, *X*. s.l. *muraokai* and *X*. s.l. *microchira* from two areas were pooled.

Taxon	n	Mean δ^{15}N value	95% CI	Mean δ^{15}N value minus 95% CI	Mean δ^{15}N value plus 95% CI
Xylophaga washingtona	4	7.2	0.56	**6.64**	7.76
Xylophaga dorsalis	2	6.3	1.8	**4.5**	8.1
Abditoconus heterosiphon	4	3.7	0.48	**3.2**	4.2
Xylophaga alexisi	4	4.4	1.27	**3.1**	5.7
Xylophaga s.l. *muraokai*	5	1.5	0.68	0.82	2.2
Xylopholas crooki	2	1.5	0.72	0.8	2.2
Xylophaga s.l. *zierenbergi*	42	−0.035	0.32	−0.36	0.29
Xylophaga s.l. *microchira*	4	−0.072	1.83	−1.9	1.8
Xylophaga oregona	3	1.5	4.0	−2.5	5.5

Bolded are significantly elevated δ^{15}N values with a lower CI > 3.

The specimens analyzed that were preserved by different chemical treatments (**Table 1**) did not significantly differ in either δ^{13}C or δ^{15}N isotopic values from specimens reported in the literature that had been frozen (noted in **Table 3**) prior to isotopic analyses (for δ^{13}C isotopic values $W = 0.514$; $p > 0.05$; for δ^{15}N isotopic values $W = 0.543$; $p > 0.05$).

Our Random Forest analyses found the most important variable associated with δ^{15}N signatures was clade membership, followed by depth, δ^{13}C value, wood type, taxon and lastly preservation. Given that preservation type covaried strongly with species, evidence for preservation type affecting our results appears to be lacking. Our phylogenetically corrected ANOVAs of *Xyloredo nooi*, *Xylophaga dorsalis*, *X. washingtona, X.* s.l. *zierenbergi*, *X.* s.l. *muraokai*, *A. heterosiphon*, and *Xylopholas crooki* failed to find significant differences in δ^{15}N or δ^{13}C values between clades after phylogenetic correction in multiple repeated randomized samplings of the phylogenetic tree. These groups did not statistically differ from one another after accounting for shared evolutionary history.

Xylophaga dorsalis Clade

In addition to the three members of the *X. dorsalis* clade with significantly elevated δ^{15}N values (**Table 2**), the fourth member of the clade considered, *X. oregona*, had a mean δ^{15}N value of 1.5 ± 1.6‰, among the highest of the remaining species considered (**Table 1**), with the largest standard deviations (**Table 1**) and much larger confidence intervals (**Table 2**).

Contrary to our predictions for strict xylophagy, the δ^{13}C values of the fecal chimneys of *X. dorsalis* (mean = −26.9 ± 1.1‰; $n = 8$) were intermediate between those of wood (−28.1 ± 0.4‰ $n = 5$) and siphon tissue (−21.8 ± 0.03‰; $n = 2$) (**Supplementary Table S3**). Consistent with our predictions, the tissues (mean δ^{13}C −22.4 ± 0.2‰; $n = 3$) of *X. oregona* were enriched relative to the wood (−24.3 ± 0.2‰; $n = 3$) and the chimneys were depleted (mean δ^{13}C −25.5 ± 0.6‰; $n = 5$) (**Supplementary Table S3**).

Relationship of Depth to Stable Nitrogen Isotopic Signature

The species' collection depths were not significantly correlated with their mean δ^{15}N values ($r = 0.394$; $n = 13$; $p > 0.05$). Removing the extreme shallow-water (<500 m) species (*X. dorsalis* and *X. washingtona*) and recalculating did not discover a significant relationship ($r = 0.467$; $n = 11$; $p > 0.10$). Kruskal-Wallis tests found depth to be significantly linked to δ^{15}N ($p < < 0.05$) when individuals rather than species means were considered.

Within Species Variation

The δ^{13}C isotopic values of six collections of 42 specimens of *Xylophaga* s.l. *zierenbergi* ranged from -26.4 to −21.5‰, exceeding the combined range of all other specimens considered (**Figure 1**). The most depleted δ^{13}C isotopic values of *X.* s.l. *zierenbergi* were from those removed from bark-covered logs of *Pinus pinea* and *Quercus agrifolia* (mean −25.7‰). The borers in the *P. pinea* deployment also included seven of the eleven most enriched in δ^{15}N values (mean 1.6 ± 0.6‰) of the 42 specimens of *X.* s.l. *zierenbergi*. Even with these outstanding specimens, the δ^{15}N isotopic values of this species ranged from −2.3 to 2.3‰, within the predicted range of freshly fixed nitrogen (**Supplementary Table S2**). The δ^{13}C values of *X.* s.l. *zierenbergi* from a single ginkgo log spanned 4.0‰ (**Figure 2**). The δ^{13}C values of specimens from deployed spicebush sticks ranged from near −25 to −22.5‰; they also showed a comparatively wide range of δ^{15}N values (−2.25 to 1.0‰).

DISCUSSION

The stable isotopic signatures of six of ten xylophagaid species we considered are entirely consistent with predicted stable isotopic signatures of strict xylophages; they eat the wood that they bore and use nitrogen fixed by bacteria. The other four species (*X. dorsalis, X. washingtona, X. alexisi* and *A. heterosiphon*) have δ^{15}N values significantly enriched compared to those we predict characterize nitrogen fixed by bacteria; they are the focus of our discussion. Although supporting data are required, we offer a hypothesis of filter feeding.

Clade Membership and Preservation Effects

Members of the *X. dorsalis* clade, *X. dorsalis, X. washingtona,* and *X. alexisi* have mean δ^{15}N values from 4.4 to 6.5‰; these significantly exceed those predicted for bacterial nitrogen fixation. Comparable data for members of this clade have been reported previously: Bernardino et al. (2010) plotted three

TABLE 3 | Literature data for $\delta^{13}C$ and $\delta^{15}N$ of Xylophagaidae.

Taxon	Depth (m)	Location	Wood type, Deployment duration, preservation	Mean Tissue $\delta^{13}C$ ‰ (std dev) n	Mean $\delta^{15}N$ ‰ (std dev)	Tissue C/N ratio	Wood $\delta^{13}C$ (std dev) n	Wood $\delta^{15}N$ (std dev) (n)	Wood C/N	References
Xylophaga sp. Wood A	150–250	34° 4.26' N 136° 18.06' E	Wild wood, frozen	−26.2 (1.57) *n* = 2	4.4 (0.07)	∼ 7	−27.1 (0.01) *n* = 2	4.17 (0.61)	120	Nishimoto et al. (2009)
Xylophaga sp. Wood C	150–250	34° 25.86' N 138° 24.66' E	Wild wood, frozen	−24 (0.26) *n* = 7	3.8 (0.18)	∼7	−25.7 *n* = 1	1.02	138	Nishimoto et al. (2009)
Xylophaga washingtona	1670	33° 27'N 119° 22'W	Fir, 5.5 yr, ?	−24 *n* = 1 −22.0 *n* = 1	0.2 *n* = 1 5.0 *n* = 1	0.75	−24.3 (0.2) *n* = 2	5.5 (0.6)	–	Bernardino et al. (2010)
Xylophaga s.l. *atlantica* ♀♀	2279	36°13.745'N 33°54.05'W	Pine, 414 days, frozen	−21.7 (0.3) *n* = 3	4.6 (0.5)	5.3 (0.3)	−23.1 (0.1) *n* = 3	–	159.6 (17.3)	Gaudron et al. (2016)
Xylophaga s.l. *atlantica* dwarf ♂♂	2279	36°13.745'N 33°54.05'W	Pine, 414 days, frozen	−20.2 *n* = 30 pooled	6.4	3.8	−23.1 (0.1) *n* = 3	–	159.6 (17.3)	Gaudron et al. (2016)
Xylophaga globosa	101–324	41° 48'S 72° 48'W	Wild wood, ?	−23.5 (0.8) *n* = 7	9.0 (5.1)	3.6 (0.4)	? −24.6 (0.7) *n* = 2	9.2 (0.3) 0.7 (0.2)	? 27.2 (6.8)	Zapata-Hernández et al. (2016)
Xyloredo teramachii	276	24° 57.19' N 125° 57.29'E	*Zelkova serrata*, 13 mo, freeze-dried	−27.5 (0.2) *n* = 4	3.2 (1.6)	–	−27.4 (0.3) *n* = 3	−0.2 (1.2)	554.3	Yamanaka et al. (2015)
Xyloredo teramachii	276	24° 57.19' N 125° 57.29'E	*Zelkova serrata*, 13 mo, freeze-dried	−26.6 (0.8) *n* = 3	3.8 (1.1)	–	−27.3 (0.3) *n* = 3	−0.3 (0.4)	554.3	Yamanaka et al. (2015)
Xyloredo teramachii	500	24° 45.00' N 125° 44.99'E	*Zelkova serrata*, 22 mo, freeze-dried	−25.8 (0.8) *n* = 3	2.3 (0.8)	–	−28 (1.7) *n* = 3	0.8 (1.6)	509.1	Yamanaka et al. (2015)
Xyloredo teramachii	1000	24° 35.01' N 125° 45.51'E	*Zelkova serrata*, 22 mo, freeze-dried	−25.7 (1.1) *n* = 4	0.8 (1.0)	–	−28.3 (0.7) *n* = 3	−1.1 (1.7)	685.6	Yamanaka et al. (2015)

Reported are taxon, depth, locality, wood type, deployment duration if applicable, mean tissue $\delta^{13}C$ (standard deviation) number, mean tissue $\delta^{15}N$ (standard deviation), tissue C/N ratio, mean wood $\delta^{13}C$ (standard deviation) number, mean wood $\delta^{15}N$ (standard deviation) number, wood C/N ratio and the literature reference. – indicates the data were not reported; ? indicates the reference was unclear.

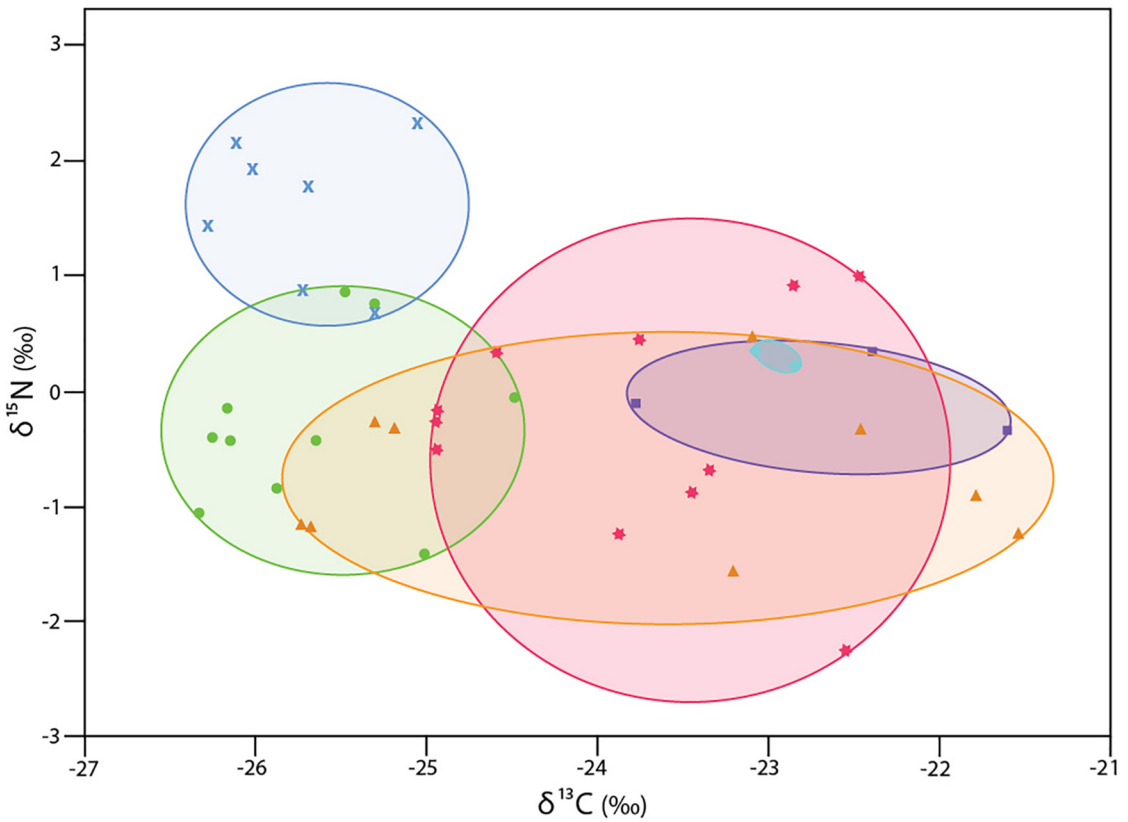

FIGURE 2 | $\delta^{13}C$ and $\delta^{15}N$ values for 42 specimens of *Xylophaga* s.l. *zierenbergi* from six deployments. wood bundles (WB) except aqua diamonds: from Douglas fir on Endeavour Segment, Juan de Fuca Ridge at 2211 m depth (Voight, 2007); all others are from deployments near Monterey Canyon at 3100 m depth (Judge and Barry, 2016): blue crosses bark-covered pine log (WB17); green circles bark-covered oak log (WB23); orange triangles gingko logs (WB26); pink stars spicebush sticks (WB19); purple squares island ironwood (WB32).

individuals of *X. washingtona* as having an average $\delta^{15}N$ signature near 4.9‰; Zapata-Hernández et al. (2016) reported *X. globosa*, assigned to this clade due to its morphology (Turner, 1955), to have $\delta^{15}N$ values of 9 ± 5.1‰. These data and the fact that most of these specimens were collected from shallow, likely more productive depths suggest that these xylophagaids may filter feed. The *X. dorsalis* clade has a significantly shallower distribution than do other members of the family (Voight, 2008).

One member of the clade, *Xylophaga alexisi*, violates the depth generality. From 4626 m depth in the Cape Verde Abyssal Plain, it has a mean $\delta^{15}N$ value of 4.4‰, despite the predicted scarcity of plankton at the abyssal collection depths. A simultaneously deployed near-bottom sediment trap, however, documented that the deployment re-suspended abyssal sediments (Khripounoff et al., 1998); perhaps these xylophagaids fed on these particles. Deep-sea nitrogen particulates may have comparatively high $\delta^{15}N$ signatures (Sigman and Casciotti, 2001). Globally, deep-sea heterotrophic animals have average $\delta^{15}N$ isotopic values from 10 to 13‰ depending on latitude (reviewed in Parzanini et al., 2019). Montoya et al. (2002) found POM $\delta^{15}N$ increases rapidly to 6–8‰ in the first 300 m in the tropical Atlantic. A small amount of abyssal sediment may enrich ^{15}N values to a greater degree than would be expected at shallow depths.

Limited behavioral data may support the hypothesis of filter feeding in the *X. dorsalis* clade. These taxa share an otherwise unique excurrent siphon that is truncated relative to the incurrent siphon. The incurrent siphon can extend over a cm beyond the wood (Purchon, 1941) into the water column, as *in situ* photos show (Bernardino et al., 2010, Figure 2b; Romano et al., 2014, Figure 2). Siphonal extension was thought to ensure access to well-oxygenated water, away from dense populations with the numerous fecal chimneys in the wood. Siphonal extension could, however, also facilitate filter feeding. As dense populations bore the wood, some individuals might secure more resources from the water column than from the remaining wood. Perhaps the extremely high variation in stable isotopic $\delta^{15}N$ signatures we document in individuals of *X. oregona* from an unusually dense population is indicative of such a scenario.

An alternate explanation for the enriched $\delta^{15}N$ signatures of the *X. dorsalis* clade is vertical transmission of a clade-specific symbiont that used another source of nitrogen. Available data, however, argue against vertical transmission of symbionts. Pyrosequencing revealed that two symbionts from three individuals of *X. dorsalis* (as sp. A) share 95% genetic similarity to those known from shallow-water teredinids

(Fagervold et al., 2014). This supports environmental acquisition of symbionts rather than vertical transmission.

The fourth species identified here as an outlier, *A. heterosiphon*, is the only species to have a mean $\delta^{13}C$ value ($-25.3 \pm 0.15‰$) more depleted than is the wood ($-23.9‰$) it bored, and apparently consumed. Its mean $\delta^{15}N$ signature of 3.7‰ is also significantly enriched compared to predictions for nitrogen fixed by bacteria. This species has complete siphons which are covered by a periostracal cone (Voight, 2007), seemingly eliminating the possibility of filter feeding. Perhaps this species exploits wood-fall associated sulfide-oxidizing bacteria (e.g., Kalenitchenko et al., 2018). Its $\delta^{13}C$ values are depleted relative to those of *X.* s.l. *microchira* that were collected during the same cruise from the same type of wood deployed at the same time and preserved in the same way with the same chemicals.

Different chemical preservatives minimally affect the stable isotopic signatures discussed here. The comparatively high variation in $\delta^{13}C$ values in wood that is likely to be reflected in the $\delta^{13}C$ values of strictly xylophagous bivalves (**Figure 2**), and the fact that stable nitrogen isotopic values are typically more robust to preservatives than are $\delta^{13}C$ values (Kaehler and Pakhomov, 2001; Ogawa et al., 2001; Sarakinos et al., 2002; Syväranta et al., 2008, 2011; Rennie et al., 2012; González-Bergonzoni et al., 2015), likely contributed to this result.

The Effects of Wood

The wood substrate into which the animals bore also appears to affect their $\delta^{13}C$ values. Specimens of *Xylophaga zierenbergi* removed from bark-covered pine and oak logs have distinctly depleted $\delta^{13}C$ values compared to conspecifics from the wood of spice bush, island ironwood, and Douglas fir. Those from logs of *P. pinea,* identified by Judge and Barry (2016), also have relatively enriched $\delta^{15}N$ values. Why these seemingly anomalous values are present remains open to conjecture and may offer an attractive area for future research. The $\delta^{13}C$ value of *P. pinea* reported in the literature is relatively depleted, −26.1 and −27‰ (Battipaglia et al., 2010; Sarris et al., 2013), but specimens of *X.* s.l. *zierenbergi* from both pine and the oak have relatively depleted $\delta^{13}C$ values. Perhaps notably, both the pine and oak logs were bark-covered; the bark may affect the wood, or its microbial community.

The $\delta^{15}N$ values of five of seven specimens from *P. pinea* are enriched relative to conspecifics, although they remain within our predicted range for nitrogen fixed by bacteria. Specimens removed from spice bush wood show more variation in the $\delta^{15}N$ values than do those from other substrates (**Figure 2**). Wood itself cannot be responsible for enriching the $\delta^{15}N$ values as its nitrogen content is too low to do so. Other factors such as oxygen levels possibly mediated by the bark covering could have influenced these results. Could such factors also have resulted in the enriched $\delta^{15}N$ values of the three members of the *X. dorsalis* clade? Given that the dramatically and consistently enriched $\delta^{15}N$ values of three species of that clade derive from wild wood falls and artificial deployments in the North Atlantic and the North Pacific from depths of 18 to 4626 m, it seems unlikely but proof is lacking. In addition, the $\delta^{13}C$ values of the *X. dorsalis* clade are generally not depleted, being directly comparable to most specimens of *X.* s.l. *zierenbergi* and to predictions for strict xylophagy.

Xylophagy: A Flawed Conclusion?

The implicit view that all xylophagaids are strict xylophages has uncertain origins. Purchon (1941) reported reduced palps in *X. dorsalis* and the poor sorting potential of their gills (Distel and Roberts, 1997). Reduced palps do characterize bivalves that host chemosynthetic bacteria on their gills (Roeselers and Newton, 2012), but gill endosymbionts do not preclude filter feeding. Bivalves of *Lucinoma borealis* and mussels of *Bathymodiolus* are among the chemosynthetic bivalves documented to feed by both means; the lucinids are estimated to obtain about half of their carbon through filter feeding (Dando et al., 1986; Page et al., 1990). Reduced palps appear to poorly predict filter feeding in wood borers, despite statements to the contrary (Saraswathy and Nair, 1971). In *T. navalis*, the paired dorsal palps are inconspicuous and the ventral ones are reduced to slightly raised ciliated patches (Lazier, 1924). Saraswathy and Nair (1971) cited Lazier's (1924) report but omitted his mention that the palps' function is "retained in *Teredo*" (p. 458), although whether they effectively controlled the plankton stream was doubtful (Lazier, 1924). Isotopic data indicate that *T. navalis* secures most of its carbon through filter feeding (Paalvast and van der Velde, 2013). The stable isotopic signatures of *B. carinata,* another teredinid with small palps (Saraswathy and Nair, 1971 as *B. indica*, synonymy following Turner, 1966), indicate that it relies entirely on xylophagy (Charles et al., 2018). The small palps of *X. dorsalis* may limit sorting, but captive individuals regularly expel pseudofeces through the incurrent siphon (JRV oight unpub data), which may indicate they exert some selection.

The scarcity of alternative food sources in the deep sea was cited by Distel and Roberts (1997) as limiting xylophagaid filter feeding. However, the species they examined had been collected at 60 and 80–100 m depth. Distel and Roberts (1997) reported few phytoplankton or microorganisms in the gut of the two species of *Xylophaga* they examined, but the animals had been maintained in "seawater tanks," potentially without a source of plankton, for up to 2 weeks before their examination; whether they examined the cecum, which has few microbes in teredinids, or the intestine, which hosts many more microbes in teredinids (Betcher et al., 2012), was not stated.

The nitrogen isotopic data cited as supporting the hypothesis of filter feeding can be subject to interpretation. A case in point is the elevated $\delta^{15}N$ signature of dwarf males of *Xylophaga* s.l. *atlantica* that was interpreted as evidence of filter feeding on fecal matter and by-products of boring in a dense population (Gaudron et al., 2016). An alternate explanation for these data is that they might reflect dwarf males parasitizing the autonomously boring female to which they attach. A review of parasitic interactions (Thieltges et al., 2019) found that parasites rarely exhibit a standard trophic shift of $\delta^{15}N$ 3.4‰. The trophic shift in $\delta^{15}N$ values seen in host-parasite interactions is often much lower, averaging 1.7‰; the nature of the parasitic interaction is likely to strongly impact that value. How the dwarf males access female resources remains conjectural; in this clade, dwarf males attach to the dorsal shell (Voight et al., 2019) rather than to

the tissues of the autonomous borers as in other clades, such as *Feaya dostwous* (Voight, 2016). The high mean δ^{15}N values (4.6 ± 0.5‰) of the autonomously boring females *X.* s.l. *atlantica* may reflect the influence of nearby hydrothermal fluid flow (Gaudron et al., 2016).

In a study that included sulfur isotopic data of the bivalves and wood, and amino acid δ^{15}N analyses, Yamanaka et al. (2015) report xylophagy by individuals of *Xyloredo teramachii* in deployments made at four depths. Yamanaka et al. (2015) used compound-specific isotopic analyses and patterns observed in marine nitrogen-fixing cyanobacteria, and terrestrial plants (Chikaraishi et al., 2009) to conclude that the bivalves were entirely xylophagous. Although their standard deviations overlapped, the mean δ^{15}N values of the bivalves at each depth are significantly and negatively correlated with depth ($r = -0.972$; $n = 4$; $p < 0.05$). A significant correlation would be expected if the animals were to opportunistically filter feed in shallower, more productive waters.

The intraspecific, and intra-deployment, variation in δ^{13}C signatures among specimens of *Xylophaga* s.l. *zierenbergi* may warn investigators seeking to use stable isotopic analyses of xylophagaids in food web studies. The points representing *X.* s.l. *zierenbergi* from gingko generally form two clusters, perhaps indicative differences between the heartwood and sapwood exposed on that log (J. Judge, unpub data). The origin of each xylophagaid might strongly influence its stable isotopic signatures. Multiple xylophagaids to be analyzed should be sampled from different parts of the wood, and matched with wood samples from the same specific area, to test if intra-wood differences affect the results. Such within-wood differences are consistent with the observed 4‰ range in δ^{13}C values of a single species in a single deployment.

The entrenched idea of strict xylophagy and the few comparable data available from other xylophagaids may have led to elevated stable isotopic signatures being discounted. Additional study of correctly identified taxa, collection and analysis of samples of particulate organic matter and sediment associated with the bored wood-fall are required to fully test the hypothesis that mixotrophy exists in xylophagaids. Although filter feeding may be opportunistic and not necessarily occur in every habitat or in every taxon, assuming that wood is the sole source of energy available to wood-fall communities may be fallacious. The amount of unknown variation in the composition of wood-fall communities (McClain and Barry, 2014; Judge and Barry, 2016) argues that assumptions should be re-examined.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

JV: conceptualization, funding acquisition, resources, visualization, writing original draft preparation, review, editing, and revision. JC: statistical analyses, editing, and revision. RL: conceptualization, data curation, formal analysis, investigation, project administration, resources, validation, and writing – review and editing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2020.00050/full#supplementary-material

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