NOTE

A synthesis of tissue-preservation effects on carbon and nitrogen stable isotope signatures

Helen C. Sarakinos, Michael L. Johnson, and M. Jake Vander Zanden

Abstract: Stable-isotope analysis is a powerful method for characterizing flows of mass and energy through ecosystems. Long-term food-web studies using stable isotopes are valuable but rare because the required samples are not readily available. We examine the feasibility of using preserved specimens from natural-history collections as a source of long-term data for food-web studies and test whether chemical preservation affects the stable-isotope signature of tissues. We experimentally determined the effects of tissue preservation and fixation with 75% ethanol and 10% formalin, respectively, on $\delta^{13}C$ and $\delta^{15}N$ of three aquatic consumers: Sacramento sucker, *Catostomus occidentalis*, Asian clam, *Corbicula fluminea*, and a caddisfly, *Hydropsyche* sp. Using both our results and previously published literature results, we characterize preservation effects across many different consumer taxa including invertebrates, fish, and birds. Overall, only formalin fixation systematically affected isotope signature, causing an average depletion of 1.65‰ in $\delta^{13}C$, a bias that can easily be corrected for prior to interpreting data. Preservation affected mean $\delta^{15}N$ values with far lower frequency and magnitude, although variability increased with preservation for some taxa but not others. These findings suggest that preserved specimens may be used for stable-isotope analysis and open up the possibility of using archived collections to reconstruct food webs and biogeochemical changes at scales of tens to hundreds of years.

Résumé : L'analyse des isotopes stables est une méthode puissante qui permet de caractériser les flux de masse et d'énergie dans les écosystèmes. Les études à long terme des réseaux alimentaires à l'aide d'isotopes stables sont précieuses, mais rares, car les échantillons requis ne sont pas facilement disponibles. Nous avons examiné la faisabilité d'utiliser des spécimens conservés dans les collections d'histoire naturelle comme source de données à long terme pour les analyses de réseaux alimentaires et nous avons vérifié si les agents de conservation chimique utilisés affectent les signatures d'isotopes stables des tissus. Nous avons déterminé expérimentalement les effets de la conservation et de la fixation dans l'éthanol 75 % et le formol 10 % sur $\delta^{13}C$ et $\delta^{15}N$ chez trois consommateurs aquatiques : le meunier de Sacramento, *Catostomus occidentalis*, la moule asiatique, *Corbicula fluminea*, et le trichoptère, *Hydropsyche* sp. Nos résultats et des travaux antérieurs dans la littérature nous ont permis de décrire les effets de la conservation chez un grand nombre de taxons de consommateurs, dont des invertébrés, des poissons et des oiseaux. En général, seule la fixation au formol affecte systématiquement la signature isotopique, causant une réduction de 1,65 ‰ de $\delta^{13}C$, une erreur qui peut facilement être corrigée avant l'interprétation des données. La conservation affecte les valeurs moyennes de $\delta^{15}N$ beaucoup moins fréquemment et moins fortement, bien que la variabilité augmente avec la durée de la conservation chez quelques taxons, mais pas chez d'autres. Ces résultats laissent espérer que des spécimens de collection pourront éventuellement être utilisés dans les analyses d'isotopes stables; on peut donc envisager la possibilité de se servir des collections de musée pour reconstruire les réseaux alimentaires et les changements biogéochimiques sur des échelles de dizaines à des centaines d'années.

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Introduction

Stable-isotope analysis is a powerful method for characterizing flows of mass and energy through ecosystems because the stable-isotope composition of a consumer's tissues is related to that of its food (DeNiro and Epstein 1978, 1981; Minagawa and Wada 1984). Carbon-isotope ratios ($\delta^{13}C$) change little from prey to predator, but vary among primary producer groups and can thus acts as tracers of carbon source (Vander Zanden and Rasmussen 2001). Nitrogen-isotope ratios ($\delta^{15}N$) exhibit consistent enrichment of 3–4‰ from prey to predator (Vander Zanden and Rasmussen 2001), providing a measure of a consumer's trophic position (Minagawa and Wada 1984; Cabana and Rasmussen 1994).

Typically, stable-isotope studies have provided qualitative descriptions of trophic interactions in individual ecosystems. Yet the importance of food-web studies at larger spatial and temporal scales has been emphasized in the recent literature (Pimm 1991; Polis and Winemiller 1996). Recent developments in baseline correction methods have facilitated comparative studies of food-web structure at broader spatial scales (Cabana and Rasmussen 1996; Vander Zanden and Rasmussen 1999). In contrast, studies of long-term trends in food webs using stable-isotope analysis have rarely been undertaken because of the lack of systematic, long-term sampling at a given locale. Natural-history museums and universities often house such site-specific collections in a preserved state. If chemical preservation has no effect, or a predictable effect, on stable-isotope composition of tissues, retrospective analysis of archived samples will be a promising tool for long-term studies of food-web and biogeochemical processes at broader time scales of decades to centuries.

In this study we experimentally quantify effects of tissue preservation on $\delta^{13}C$ and $\delta^{15}N$ signatures for three aquatic consumer species. Using both our results and previously published literature results, we characterize overall preservation effects and discuss some implications of the results for long-term food-web studies.

Our experiments

We tested for effects of preservation in 70% ethanol (EtOH; a preservative) and 4% formaldehyde (10% formalin; a fixative). Effects were tested on isotope ($\delta^{13}C$ and $\delta^{15}N$) signatures of three freshwater organisms: a fish, *Catostomus occidentalis*, an aquatic insect, *Hydropsyche* sp., and a mollusk, *Corbicula fluminea*. Organisms were collected in October 1999 and immediately processed in the laboratory. Dorsal white-muscle tissue was removed from *C. occidentalis*. *Corbicula fluminea* tissue was removed from the shell prior to preservation. *Hydropsyche* sp. specimens were preserved whole. All species underwent two preservation treatments and a control treatment (–25°C storage). Treated tissues were sampled at 3 days, 3 weeks, and 3 and 6 months. Fish and invertebrate samples were dried at 75°C for 48–72 h and pulverized using a mortar and pestle. The powder was packed into 4 × 6 mm tin capsules for carbon- and nitrogen-isotope analysis.

Stable-isotope analysis

Stable-isotope analysis was conducted on a continuous-flow isotope-ratio mass spectrometer (dual-inlet Europa 20/20, PDZ Europa, Crewe, England). Carbon and nitrogen stable isotope ratios are expressed in delta (δ) notation, defined as parts per thousand (‰, or per mil) deviations from a standard material. The standard is atmospheric nitrogen for $\delta^{15}N$ and Pee Dee belemnite (PDB) limestone for $\delta^{13}C$; both standards have a δ value arbitrarily set at 0‰. Delta^{13}C or $\delta^{15}N = [(R_{sample}/R_{standard}) - 1 \times 1000]$, where $R = {}^{13}C/{}^{12}C$ or ${}^{15}N/{}^{14}N$. A more positive "δ" value is isotopically enriched, meaning that the sample contains proportionally more of the heavy stable isotope (^{13}C or ^{15}N). Twenty-five percent of the samples were analyzed in duplicate; the mean error of our sample replicates was 0.09‰ for $\delta^{13}C$ and 0.15‰ for $\delta^{15}N$.

Data analysis

We used paired *t* tests to test for the effect of EtOH and formalin preservation on $\delta^{13}C$ and $\delta^{15}N$ of fish and invertebrate tissues. We used analysis of variance (ANOVA) to test for effects of species and time and species-by-time interactions. Stated values represent the difference between control and treatments; positive values indicate that preservation resulted in isotopic enrichment, while negative values indicate isotopic depletion. Significance was based on Type III sums of squares. Data were analyzed using SPSS for Windows version 10.0 (SPSS Inc.). All stable-isotope values reported below are calculated as the average over the 6-month duration of the experiment, i.e., the average of the four test dates.

Effects of EtOH and formalin

Only EtOH-preserved *C. fluminea* $\delta^{13}C$ was enriched by 2.2‰ ($p = 0.007$) relative to control (Fig. 1*a*; Table 1). EtOH preservation had a small though significant effect on $\delta^{15}N$ of *C. occidentalis* (0.37 ‰, $p < 0.001$) and *C. fluminea* (–0.39‰, $p = 0.03$) (Fig. 1*b*; Table 1). *Hydropsyche* sp. $\delta^{15}N$ was not significantly affected (Fig. 1*b*; Table 1).

Formalin fixation affected the three species differently (Fig. 1*a*). Overall, formalin-preserved *Hydropsyche* sp. and *C. occidentalis* tissue $\delta^{13}C$ was depleted relative to control (–0.75 and –1.33‰, respectively, $p = 0.00$; Table 1). *Corbicula fluminea* $\delta^{13}C$ was enriched relative to control (0.67‰, $p = 0.2$), though this value was not statistically significant and the slope of change fluctuated dramatically over time (Fig. 1*a*; Table 1). Formalin affected samples rapidly (within 3 days to 3 weeks) except for *Hydropsyche* sp., where the effect appeared to increase with time (Fig. 1*b*). No significant species-by-time interactions were observed. Formalin preservation had little effect on $\delta^{15}N$. Only *C. fluminea* tissue $\delta^{15}N$ was significantly depleted, by 0.48‰ ($p = 0.002$); $\delta^{15}N$ of *Hydropsyche* sp. and *C. occidentalis* did not differ from control (Fig. 1*b*; Table 1).

The broader context

Preservative effects on $\delta^{13}C$

Combining the results from our experiments and those from previously published studies, we present the overall impacts of EtOH and formalin on carbon-isotope ratios in animal tissues (Table 1). Overall, EtOH had significant effects on $\delta^{13}C$ in 3 of 9 studies; only *Drosophila melanogaster* and *C. fluminea* showed significant, though opposite, shifts in $\delta^{13}C$ relative to control. *Drosophila melanogaster* was depleted in $\delta^{13}C$ by 1.3‰; *C. fluminea* was enriched in $\delta^{13}C$ by

Table 1. Effects of EtOH and formalin preservation on tissue $\delta^{13}C$ and $\delta^{15}N$ signatures.

	Scientific name	Common name	$\Delta\delta^{13}C$[a]	$\Delta\delta^{15}N$[a]	Reference
EtOH	*Cotrunix japonica*	Quail			
	Egg, yolk lipid[b]		0.27	na	Gloutney and Hobson 1998
	Egg, lipid-free yolk[b]		0.08	–0.10	Gloutney and Hobson 1998
	Egg, albumin[b]		0	–0.07	Gloutney and Hobson 1998
	C. japonica[b]		–0.44	0.40	Hobson et al. 1997
	Drosophila melanogaster	Fruit fly			
	10 days[b]		–1.38*	0.17	Ponsard and Amlou 1999
	6 weeks[b]		–1.17*	0.12	Ponsard and Amlou 1999
	Catostomus occidentalis	Sacramento sucker	0.21	0.37*	This study
	Corbicula fluminea	Asian clam	2.18*	–0.39*	This study
	Hydropsyche sp.	Caddisfly	0.04	–0.21	This study
	n		18	17	
	Mean ± SE		–0.02 ± 0.24	0.04 ± 0.07	
Formalin	*C. japonica*				
	Egg, yolk lipid[b]	Quail	–0.16	n/a	Gloutney and Hobson 1998
	Egg, lipid-free yolk[b]		–2.44*	0.03	Gloutney and Hobson 1998
	Egg, albumin[b]		–2.39*	0.24	Gloutney and Hobson 1998
	C. japonica[b]		–1.78*	0.04	Hobson et al. 1997
	D. melanogaster				
	10 days[b]	Fruit fly	–2.92*	0.34*	Ponsard and Amlou 1999
	6 weeks[b]		–2.69*	0.08	Ponsard and Amlou 1999
	C. occidentalis	Sacramento sucker	–1.33*	0.16	This study
	C. fluminea	Asian clam	0.67	–0.48*	This study
	Hydropsyche sp.	Caddisfly	–0.75*	–0.121	This study
	Crangon septemspinosa	Mud shrimp	–2.05	0.35	Bosley and Wainright 1999
	Pleuronectes americanus	Winter flounder	–0.74	1.21*	Bosley and Wainright 1999
		Marine zooplankton	–2.50[c]	–1.00[c]	Mullin et al. 1984
	Neomysis intermedia	Freshwater shrimp	na	0.04	Toda and Wada 1990
	n		21	22	
	Mean ± SE		–1.65 ± 0.24	0.08 ± 0.12	

[a]Difference between control and treatment values. A positive value indicates that the treatment resulted in isotopic enrichment, a negative value denotes the opposite.
[b]No statistical information is available from this study.
[c]Samples were rinsed in distilled water to remove preservative prior to desiccation.
*$p < 0.05$.

>2‰ (Table 1). Across all studies, formalin fixation resulted in an average $\delta^{13}C$ depletion of 1.65‰ (SE = 0.24‰), although some species were not significantly affected (Table 1). Additionally, organisms that were most depleted in $\delta^{13}C$, *Cotrunix japonica* and its eggs and *D. melanogaster*, were rinsed in distilled water prior to desiccation. This step may have accelerated the breakdown of proteins, since bonds that are formed between formaldehyde and proteins are unstable and easily dissociable (Taylor 1977; Stephenson and Riley 1995).

EtOH and formalin may have affected preserved specimens through two very different mechanisms. The first is selective loss of material from the specimen during preservation. EtOH hydrolyzes lipids, while formalin hydrolyzes protein (Von Endt 1994; Hobson et al. 1997; Gloutney and Hobson 1998; Bosley and Wainright 1999). Considering that lipids are depleted in $\delta^{13}C$ relative to protein (DeNiro and Epstein 1977; Von Endt 1994; Focken and Becker 1998), the observed depletion of $\delta^{13}C$ in formalin-preserved specimens is consistent with a loss of hydrolyzed proteins. However, differences in lipid content among our three tested organisms did not explain any of the observed patterns in the effect of EtOH on $\delta^{13}C$.

The second mechanism is uptake of the preservative into the tissue. Both preservatives are carbon-based chemicals with characteristic $\delta^{13}C$ signatures. Once preserved samples are immersed, their signature may shift toward that of the preservative (Hobson et al. 1997; Gloutney and Hobson 1998; Bosley and Wainright 1999; Ponsard and Amlou 1999). In our experiment, $\delta^{13}C$ in both fish and aquatic invertebrates (–27 to –28‰) shifted toward that of the formalin medium (–32‰) following fixation (Fig. 1*b*). No such shift was evident for EtOH.

Preservative effects on $\delta^{15}N$

Findings from our experiments and from the literature (Mullin et al. 1984; Hobson et al. 1997; Gloutney and Hobson 1998; Ponsard and Amlou 1999) indicate a minimal effect of either preservative on $\delta^{15}N$ across all species (mean formalin effect: 0.10 ± 0.12‰; mean EtOH effect: 0.02 ±

Fig. 1. Effect of EtOH (*a*) and formalin (*b*) on $\delta^{13}C$ and $\delta^{15}N$ of *Hydropsyche* sp., *Corbicula fluminea*, and *Catostomus occidentalis* (●, control (frozen) values; ○, treatment values). Treatment length corresponds to the sequence in which treated samples were analyzed for isotope signature: 3 days, 3 weeks, 3 months, and 6 months. Results from two populations are shown for *Hydropsyche* sp. (populations are seperated by the vertical line) and from three individuals for *C. occidentalis* (individuals are seperated by the vertical lines). The dotted horizontal line in the $\delta^{13}C$ plots corresponds to the signature of the preservative; there is no corresponding $\delta^{15}N$ signature, since preservatives contain no nitrogen. Parentheses after treatment length in the *x* axis identify the individual populations (for *Hydropsyche*) or specimens (for *C. occidentalis*).

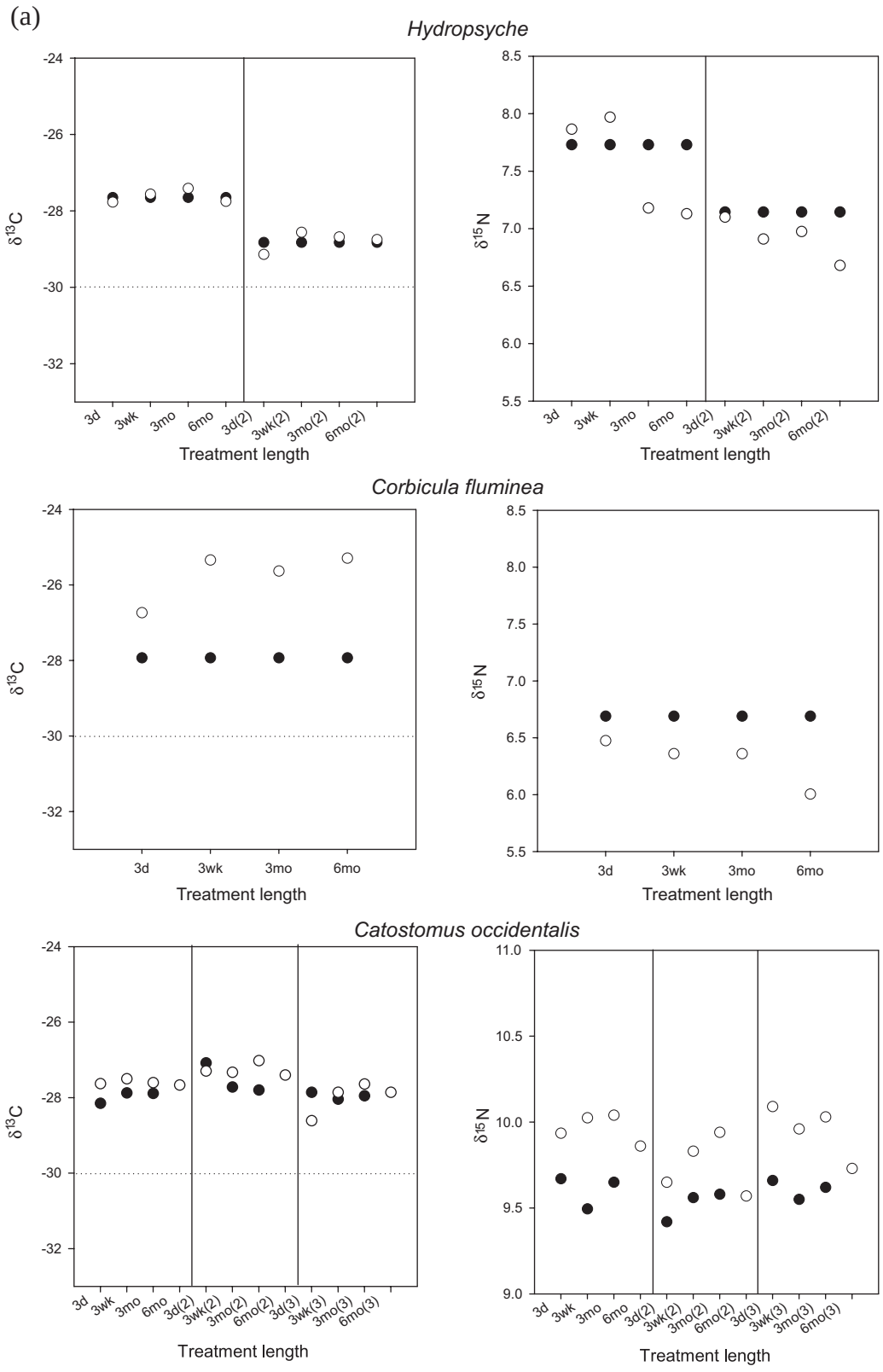
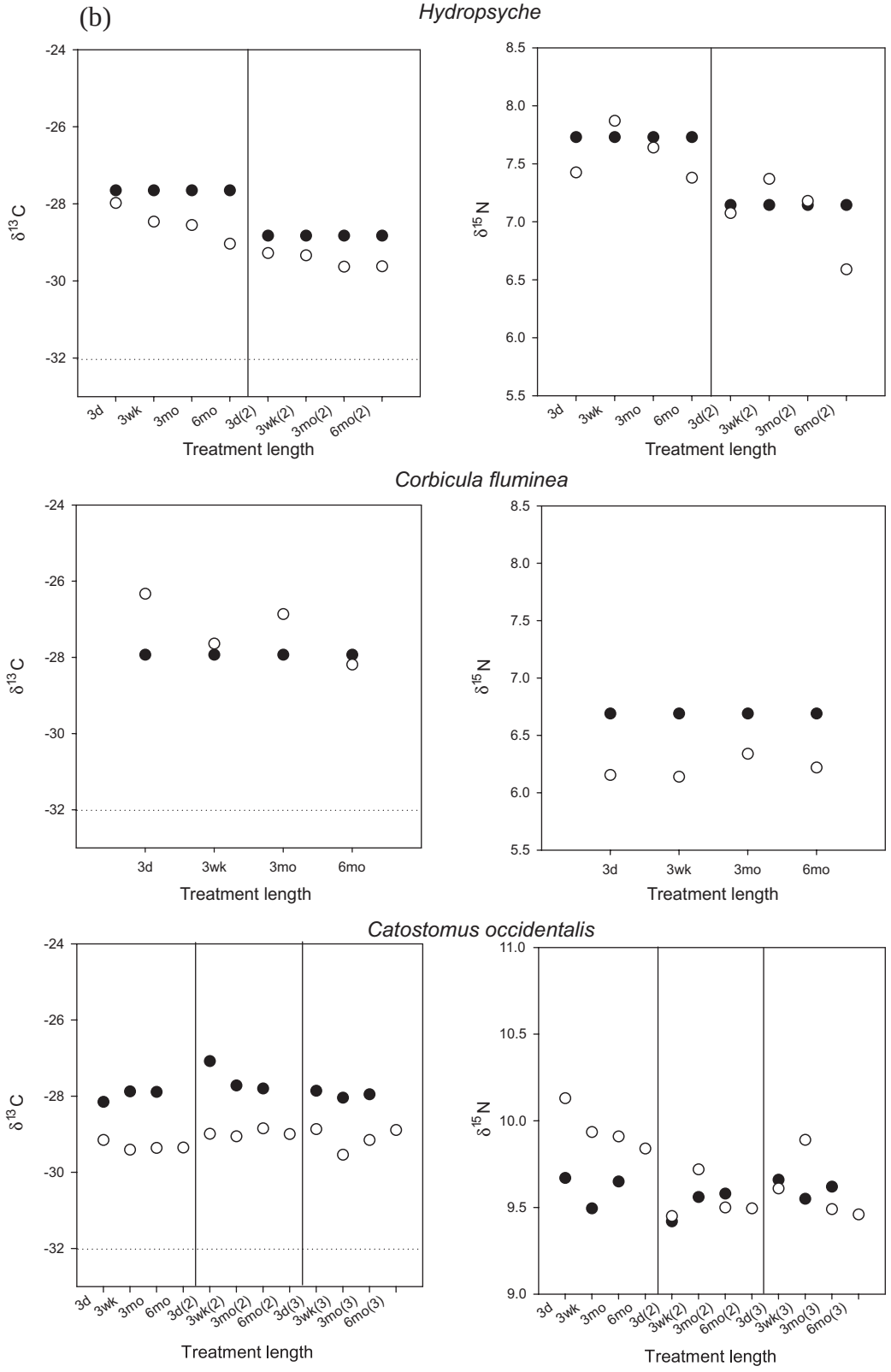


Fig. 1 (*concluded*).



0.06‰; n = 23; Table 1). While some studies did show a significant effect of preservative on $\delta^{15}N$, shifts in $\delta^{15}N$ did not exceed 0.5‰ except in formalin-preserved *Pleuronectes americanus*, which was enriched by 1.4‰. Because both EtOH and formalin contain no nitrogen, any change in $\delta^{15}N$ following preservation would be due to tissue hydrolysis and leaching, not to uptake of the N signature from the preservative. Bosley and Wainright (1999) observed that variability in $\delta^{15}N$ was greater in preserved *Crangon septemspinosa* than in control *C. septemspinosa* (95% confidence intervals

2.49 and 0.45, respectively). Similarly, we found that the standard deviation of $\delta^{15}N$ values for preserved *C. occidentalis* samples was higher than that for controls ($SD_{control}$ = 0.08‰, SD_{EtOH} = 0.165‰, $SD_{formalin}$ = 0.23‰).

Potential application to long-term ecological studies

Stable isotopes provide an integrative approach for measuring food-web interactions, although most food-web studies have been conducted at relatively short time scales. Longer term food-web studies can contribute valuable insights (Wainright et al. 1993), but have rarely been conducted because the required samples are not available. Natural-history museums house rich collections of animal specimens, often collected over long time periods from a given location. As our understanding of how isotope signatures are affected by preservation builds, the potential exists to use long-preserved specimens for stable-isotope analysis. Only formalin fixation systematically affected isotope signature, producing a mean depletion of 1.65‰ in $\delta^{13}C$, a bias that can easily be corrected for prior to data interpretation. The effects of EtOH appear to be of lesser magnitude but are also less consistent. While these results are promising, further research is needed to verify that preservation effects remain similar at time scales beyond those examined here (>6 months). These findings open up the possibility of reconstructing food-web and biogeochemical changes over time scales of decades to centuries. This approach can be used to address a number of important questions, including the impacts of species invasion (Vander Zanden et al. 1999) and movement of contaminants through food webs (Cabana and Rasmussen 1994; Kidd et al. 1995, 1998). Current approaches used for retrospective food-web analyses are limited in their ability to resolve changes at these time scales.

Global collections of formalin/EtOH-preserved specimens currently stand at 1.5 billion and are growing at about 50 million a year (Peake 1989), constituting a significant portion of the record of the world's documented biodiversity (Von Endt 1994). Furthermore, many fields of biological research depend on the use of intact preserved specimens. Practical applications such as those presented here augment the value of natural-history collections and are a further incentive to continue the collection and maintenance of preserved archived samples of the world's biodiversity.

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