

few hours of the commencement of a cold downslope flow (Fer et al. 2001), and over longer times and greater depth, the effects of the Earth's rotation may become important. However, this will not significantly affect the result we obtained from the analysis above.

*Ilker Fer<sup>1</sup> and Ulrich Lemmin*

Laboratoire de Recherches Hydrauliques  
Dépt. Génie Civil  
Ecole Polytechnique Fédérale de Lausanne  
CH-1015 Lausanne, Switzerland

*S. A. Thorpe*

School of Ocean and Earth Science  
Southampton Oceanography Centre  
European Way  
Southampton SO14 3ZH, United Kingdom

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<sup>1</sup> To whom correspondence should be addressed. Present address: Geophysical Institute, University of Bergen, Allegaten 70, N-5007, Bergen, Norway.

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## Interpreting stable isotopes from macroinvertebrate foodwebs in saline wetlands

**Abstract**—We compared stable-isotope ( $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ) and gut-content analyses of macroinvertebrate foodwebs in saline wetlands of the Laramie Basin, Wyoming, USA. Standard assumptions of stable-isotope fractionation between trophic levels ( $<1\%$  for  $\delta^{13}\text{C}$ , mean of  $3.4\%$  for  $\delta^{15}\text{N}$ ) suggested that zygopteran (damselfly) larvae consumed mainly amphipods. However, the guts of zygopterans contained no amphipods but rather a mix of chironomid larvae and zooplankton. In all wetlands the gut contents of zygopterans indicated that they were secondary consumers (trophic level 3), but enrichment of  $\delta^{15}\text{N}$  between zygopterans and their prey ( $\Delta\delta^{15}\text{N}$ ) varied from 1 to  $3.4\%$  between wetlands. In other studies, such variation in  $\Delta\delta^{15}\text{N}$  has been interpreted to mean that food-chain length differed between aquatic systems. We review alternative interpretations of variable  $^{15}\text{N}$  enrichment, namely, varying C:N ratios in food, differential enrichment between consumer species, and habitat-specific variation of  $\delta^{15}\text{N}$  at the base of foodwebs. We also suggest that variation in the timing and rates

of nitrogen cycling can affect measured  $\Delta\delta^{15}\text{N}$  both within and between foodwebs. For aquatic macroinvertebrates, we urge that stable isotopes be supplemented with independent observations to avoid incorrect conclusions about trophic pathways, trophic levels, and food-chain lengths in different ecosystems.

Macroinvertebrate foodwebs are the main link between high primary production and top consumers in wetlands. Two main tools for characterizing such foodwebs are gut-content analysis and stable isotopes. The latter method is often used, but there can be problems interpreting stable-isotope data. For example,  $\delta^{13}\text{C}$  typically increases by  $<1\%$  with each trophic transfer, so that  $\delta^{13}\text{C}$  can be used to discriminate diets derived from different sources of primary production (Peterson and Fry 1987). However, consuming a

mixture of prey types can yield an isotope signature resembling that of an unused food. Values of  $\delta^{15}\text{N}$  usually increase by 3 to 5‰ with each trophic transfer, and an average enrichment of +3.4‰ per trophic level is often assumed for aquatic foodwebs (Cabana and Rasmussen 1994; Vander Zanden et al. 1999; Post et al. 2000). However, important deviations from this standard fractionation of  $^{15}\text{N}$  can occur for the same food with different nitrogen content (Adams and Sterner 2000), among different consumer species (Macko et al. 1982), and among habitats in the same aquatic ecosystem (Vander Zanden and Rasmussen 1999). These caveats to interpreting both  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  can confound analyses of primary producer sources, trophic pathways, and food-chain lengths.

In this paper, we compare results of gut-content and stable-isotope analyses of macroinvertebrate foodwebs in shallow saline lakes. We present an example in which stable-isotope data were misleading indicators of diet and discuss several interpretations of variable  $^{15}\text{N}$  enrichment with trophic level. We found that this foodweb would be seriously misinterpreted by using stable-isotope analyses without examining gut contents.

**Methods**—Macroinvertebrates were collected in June 1999 from two oligosaline and two mesosaline wetlands near Laramie, Wyoming, USA (see Lovvorn et al. 1999). Macroinvertebrates were sampled at different times during the day over a period of 1 week. We measured  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  for three individual specimens each of zygopteran larvae (*Enallagma* spp.), chironomid larvae (Chironominae and Orthocladinae), amphipods (*Hyalella azteca*), copepods (*Diptomus* spp.), and cladocerans (*Daphnia* spp.). Samples for determining  $\delta^{13}\text{C}$  were rinsed in 10% HCl and then distilled water to remove any calcium carbonate precipitates. For zygopterans (damselflies), chironomids, and amphipods, gut contents were removed before isotope analysis. Percent dry mass of each prey type in the foregut of zygopterans was measured by counting prey items at 100× magnification and multiplying the number of each prey type by its average mass (we counted entire organisms or fragments that were sometimes grouped as a single organism). For cladocerans and copepods (no amphipods were found in zygopteran guts), we determined average mass by weighing field samples. For chironomids, we measured head capsule width with an ocular micrometer and applied the regressions of mass versus capsule width of Benke et al. (1999). Based on isotope values and the percent mass in guts for each prey type (assuming assimilation efficiencies are equal), we used mixing models to estimate the isotope signature of zygopteran prey:

$$\delta^{13}\text{C}_{\text{mixed prey}} = [\% \text{mass}_{\text{cladocerans}}(\delta^{13}\text{C}_{\text{cladocerans}}) + \% \text{mass}_{\text{copepods}}(\delta^{13}\text{C}_{\text{copepods}}) + \% \text{mass}_{\text{chironomids}}(\delta^{13}\text{C}_{\text{chironomids}})]/100, \text{ and}$$

$$\delta^{15}\text{N}_{\text{mixed prey}} = [\% \text{mass}_{\text{cladocerans}}(\delta^{15}\text{N}_{\text{cladocerans}}) + \% \text{mass}_{\text{copepods}}(\delta^{15}\text{N}_{\text{copepods}}) + \% \text{mass}_{\text{chironomids}}(\delta^{15}\text{N}_{\text{chironomids}})]/100$$

To examine  $\delta^{15}\text{N}$  enrichment of zygopterans versus their diet ( $\Delta\delta^{15}\text{N}$ ), we computed both  $\delta^{15}\text{N}_{\text{zygopterans}} - \delta^{15}\text{N}_{\text{mixed prey}}$  and  $\delta^{15}\text{N}_{\text{zygopterans}} - \delta^{15}\text{N}_{\text{chironomids}}$ . The latter difference is a computation of  $\delta^{15}\text{N}$  enrichment versus a baseline indicator organism (chironomid larvae). To avoid uncertainties in the sources and condition of organic matter entering foodwebs, recent authors have used baseline indicator organisms (Vander Zanden et al. 1999; Post et al. 2000). Rather than measuring isotopes in algae or detritus, this approach uses the  $\delta^{15}\text{N}$  (and  $\delta^{13}\text{C}$  where appropriate) of primary consumers to represent stable-isotope signatures at the base of foodwebs. These baseline consumers provide time-integrated isotope values that buffer fluctuations in the quality, assimilation efficiency,  $\delta$  values, and preliminary fractionation of plant or detrital foods. For example, zooplankton assimilate only labile particles from seston, so their tissues reflect the isotope baseline of the foodweb better than bulk seston collected on a filter does. We used chironomid larvae (Chironominae and Orthocladinae) as baseline indicator organisms because they likely integrate the most available algal and detrital foods and because they represented about 93% by mass of zygopteran diets over all four lakes.

**Results of diet determinations**—The  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values for a predator (zygopteran larvae) and four potential prey in Creighton Lake are shown in Fig. 1. Standard assumptions of stable-isotope fractionation between trophic levels in aquatic foodwebs (<1‰ for  $\delta^{13}\text{C}$ , mean of 3.4‰ for  $\delta^{15}\text{N}$ ) indicate that zygopteran larvae fed mainly on amphipods: zygopteran and amphipod  $\delta^{13}\text{C}$  were within 1‰, and zygopteran  $\delta^{15}\text{N}$  was enriched by almost exactly 3.4‰ over that of amphipods. However, in this lake, 86 food items from 30 zygopteran stomachs consisted of 24% cladocerans, 44% copepods, 29% chironomids, and 3% miscellaneous nonamphipod prey (corresponding dry mass fractions were 5% for cladocerans, 7% for copepods, and 88% for chironomids). Thus, although stable isotopes suggested that zygopterans ate mainly amphipods, they actually consumed few if any amphipods. Moreover, adhering to the standard fractionation assumptions would indicate that amphipods were a more likely diet than mixed prey. The  $\Delta\delta^{15}\text{N}$  (difference in  $\delta^{15}\text{N}$ ) for mixed prey versus zygopterans was similar to that for amphipods at about 3.4‰ (Fig. 1), but the  $\Delta\delta^{13}\text{C}$  for the true diet of mixed prey was about 2.4‰, farther from the expected difference of <1‰ than was the value for amphipods. While it is possible that the isotope values of zygopteran tissue represent amphipods consumed in the weeks prior to sampling, it is still unlikely because in these wetlands amphipods are generally too large for *Enallagma* larvae to consume (Hart and Lovvorn unpubl. data).

Any conclusions based solely on the data in Fig. 1 are suspect because four potential food items cannot be resolved with only two isotopes. A single mixing equation can be constructed for each isotope:

$$\delta_{\text{consumer}} = X + f_1\delta_1 + f_2\delta_2 + f_3\delta_3 + f_4\delta_4 \quad (1)$$

where  $\delta_n$  is the isotope value of prey  $n$ ,  $f_n$  is the mass fraction of prey  $n$  in the consumer's diet, and  $X$  is isotope fractionation between the consumer and its diet. Summing the diet fractions yields another equation:

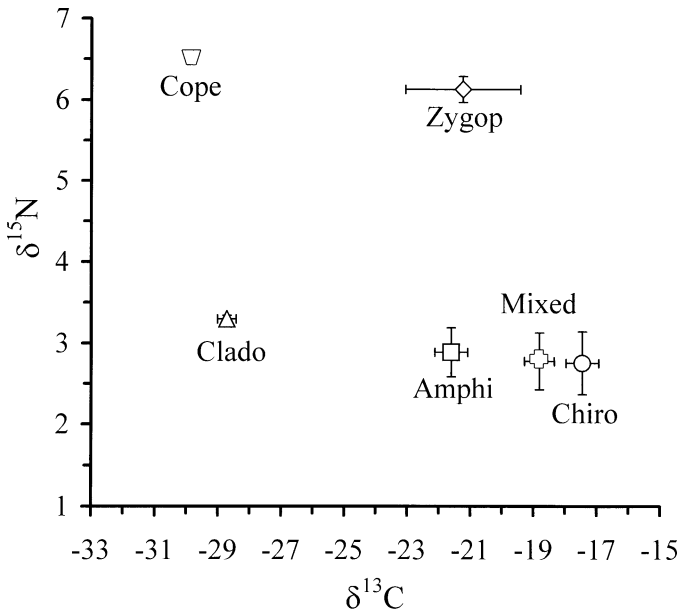


Fig. 1. Values of stable carbon and nitrogen isotopes ( $\pm 1$  SD,  $n = 3$  individual specimens per species) for predatory zygopteran larvae and potential prey in Creighton Lake of the Laramie Basin, Wyoming. Zygopterans were *Enallagma* spp., amphipods were *Hyalella azteca*, chironomid larvae were *Chironominae*, copepods were *Diatomus* spp., and cladocerans were *Daphnia* spp. Values for mixed prey were estimated from gut-content analysis and isotope values of different prey. Error bars on mixed prey reflect variability in prey isotope signatures ( $\pm 1$  SD,  $n = 3$ ) only and not error in percentage mass of gut contents. Gut-content analysis revealed that zygopterans did not feed on amphipods but on a mix of chironomid larvae, copepods, and cladocerans. Zygop = zygopteran larvae, cope = copepods, chiro = chironomid larvae, mixed = mixed prey, amphi = amphipods, clado = cladocerans.

$$f_1 + f_2 + f_3 + f_4 = 1 \quad (2)$$

In our case, using two isotopes yielded three equations (Eq. 1 for both  $^{13}\text{C}$  and  $^{15}\text{N}$ , and Eq. 2) and four unknowns ( $f_1, f_2, f_3, f_4$ ). Actually, the fractionations ( $X$ ) between diet and predator tissue for both  $^{13}\text{C}$  and  $^{15}\text{N}$  represent at least two additional unknowns. Sometimes only two isotopes can be used to infer trophic pathways if the data exhibit extreme values. For example, if zygopterans had  $\delta^{13}\text{C}$  of  $-30\text{‰}$  and  $\delta^{15}\text{N}$  of  $+10\text{‰}$ , one might reasonably infer that copepods were the main prey (Fig. 1). Extreme values often allow use of  $^{13}\text{C}$  to identify organic matter sources for primary consumers. However, for investigating trophic pathways and food-chain lengths, extreme values are less common and assumptions about fractionation ( $X$ ) more critical.

**Interpreting  $\delta^{15}\text{N}$  enrichment**—Our values for  $\Delta\delta^{15}\text{N}$  of zygopterans versus prey ranged from 1 to 3.4‰ across four wetlands (Fig. 2). Assuming standard fractionation of  $+3.4\text{‰}$   $\delta^{15}\text{N}$  per trophic transfer would indicate that zygopteran trophic levels ranged from 2.3 to 3.0. However, in all four wetlands, the gut contents of zygopterans showed that they consumed prey only at trophic level 3 (i.e., they ate only chironomid larvae and zooplankton at level 2). We readily accept the notion of noninteger trophic levels (Ulan-

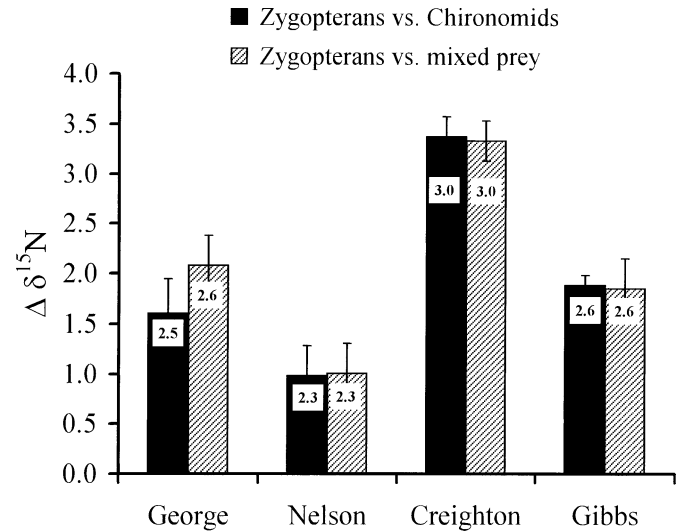


Fig. 2.  $\delta^{15}\text{N}$  enrichment ( $\pm 1$  SD,  $n = 3$  individual specimens) of zygopteran larvae versus prey ( $\Delta\delta^{15}\text{N} = \delta^{15}\text{N}_{\text{zygopterans}} - \delta^{15}\text{N}_{\text{prey}}$ ) in four saline wetlands of the Laramie Basin, Wyoming. Values in bars represent trophic levels computed as  $(2 + \Delta\delta^{15}\text{N})/3.4$ , so these values do not correspond to the ordinate scale. On average, zygopterans consumed 93% chironomid larvae by mass. Values for mixed prey were estimated from gut-content analysis and prey isotope values (see text). Error bars on mixed prey reflect variability in prey isotope signatures only and not error in percentage mass of gut contents.

owicz 1995; France et al. 1998; Post et al. 2000); however, different  $\Delta\delta^{15}\text{N}$  values among lakes did not clearly correspond to varying food-chain lengths (cf. Vander Zanden et al. 1999; Post et al. 2000). If zygopterans did consume some undetected predatory organisms then higher  $\Delta\delta^{15}\text{N}$  would result. Alternatively, zygopterans consume both chironomid tissues and gut contents (we analyzed only tissues), and assimilation of algae from chironomid guts would tend to lower  $\Delta\delta^{15}\text{N}$ . Particularly for invertebrate foodwebs, it appears that variations in  $\Delta\delta^{15}\text{N}$  ( $\delta^{15}\text{N}_{\text{organism}} - \delta^{15}\text{N}_{\text{diet}}$ ) do not always mean that the food chains have variable lengths (cf. Vander Zanden et al. 1999; Post et al. 2000). Alternative explanations in our study and others should be considered.

First, Adams and Sterner (2000) demonstrated that  $^{15}\text{N}$  enrichment varied with dietary nitrogen content for *Daphnia magna*. In laboratory experiments, these authors found a range of 0 to 6‰  $\Delta\delta^{15}\text{N}$  depending on the C:N ratio of a single algal food. However, results in Fig. 2 represent a predator-prey rather than an herbivore-algal interaction. Unlike algae in the study of Adams and Sterner (2000), chironomids in our different wetlands should have had similar C:N ratios, although inadequate nitrogen intake can affect  $\delta^{15}\text{N}$  by increasing retention of  $^{15}\text{N}$  (e.g., nitrogen starvation, Hobson et al. 1993; Gannes et al. 1997).

Second, Macko et al. (1982) showed the potential for difference in  $^{15}\text{N}$  fractionation between species of aquatic invertebrates. For two species of marine amphipods,  $\Delta\delta^{15}\text{N}$  values were consistently  $+2.3\text{‰}$  for *Amphithoe valida* and  $-0.7\text{‰}$  for *Parhyale hawaiiensis* for two algal foods. However, in our study, all zygopterans were the same or very similar species (all *Enallagma* spp.) likely to have similar

fractionations for similar foods. Even so, different fractionation between species probably can never be completely dismissed without laboratory experiments.

Third, Vander Zanden and Rasmussen (1999) found that baseline  $\delta^{15}\text{N}$  varied between habitats within the same aquatic ecosystems. In lakes on the Canadian Shield, there were systematic differences in  $\delta^{15}\text{N}$  between primary consumers in littoral, pelagic, and profundal habitats. In Fig. 2, the contributions of planktonic and epiphytic nitrogen to the  $\delta^{15}\text{N}$  of zygopteran diets is accounted for in the computation of mixed prey. However, disparate  $^{15}\text{N}$  signals between habitats within a wetland could have influenced  $\Delta\delta^{15}\text{N}$  if dietary nitrogen was not homogeneously assimilated, i.e., if nitrogen sources from one habitat were used to build tissue and nitrogen sources from another habitat were catabolized for energy. For example, zygopteran tissue in Nelson Pond (Fig. 2) might be enriched by only 1‰  $\delta^{15}\text{N}$  versus bulk diet, but enriched by 3‰ versus the source of nitrogen that was routed to tissues (*see* Gannes et al. 1997 for a thorough discussion of isotopic routing). This explanation is also difficult to rule out without laboratory experiments.

In addition to these alternative explanations for the  $\Delta\delta^{15}\text{N}$  patterns in Fig. 2, there is at least one other possible interpretation besides variable food-chain lengths. Within lakes, values of  $\delta^{15}\text{N}$  for seston and epiphyton differed by up to 3.7‰ between June and August; thus, variations in  $\Delta\delta^{15}\text{N}$  among lakes might reflect varying lag times for changing  $^{15}\text{N}$  signals to reach zygopterans. Seasonal shifts of algal  $\delta^{15}\text{N}$  could create variable  $\Delta\delta^{15}\text{N}$  between lakes in at least two ways. First, identical foodwebs will yield different  $\Delta\delta^{15}\text{N}$  values if shifts in algal  $\delta^{15}\text{N}$  occur at different times, i.e., if  $^{15}\text{N}$  shifts are out of phase. Second, foodwebs with identical pathways that experience the same shifts in algal  $\delta^{15}\text{N}$  will yield different  $\Delta\delta^{15}\text{N}$  values if nitrogen turnover rates vary for component organisms. Thus, among-lake variation in the turnover rates of chironomids or other zygopteran prey might be an important element of  $\Delta\delta^{15}\text{N}$  variation between our wetlands. From this perspective,  $\Delta\delta^{15}\text{N}$  might reflect the timing and rates of nitrogen cycling more than it reflects trophic position *per se*.

#### Simulating effects of nitrogen-cycle dynamics on $\Delta\delta^{15}\text{N}$

Because variations in the timing and rates of nitrogen cycling can theoretically affect  $\Delta\delta^{15}\text{N}$  in foodwebs, we assessed the potential magnitude of these effects. We performed computer simulations for a simple foodweb of algae  $\rightarrow$  chironomids  $\rightarrow$  zygopterans and computed  $\Delta\delta^{15}\text{N} = \delta^{15}\text{N}_{\text{zygopterans}} - \delta^{15}\text{N}_{\text{chironomids}}$  through time as algal  $\delta^{15}\text{N}$  changed. In each case,  $\delta^{15}\text{N}$  of algae shifted by 3‰ in about 60 d. This shift is comparable to what we observed, e.g., seston  $\delta^{15}\text{N}$  changed by an average of 2.5‰ between seasons in all wetlands (Hart and Lovvorn unpubl. data). Post et al. (2000) reported within-lake seasonal shifts of  $>4\%$   $\delta^{15}\text{N}$ , and Leggett et al. (2000) measured shifts of  $>5\%$   $\delta^{15}\text{N}$  in lake seston in 1.5 months. Pennock et al. (1996) demonstrated that far greater shifts in algal  $\delta^{15}\text{N}$  than these are possible over a much shorter time, depending on the concentration and form of nitrogen available ( $\text{NO}_3^-$  versus  $\text{NH}_4^+$ ). For nitrogen turnover rates, we used values reported for macroinvertebrates by Dodds et al. (2000): 0.03 per day for zyg-

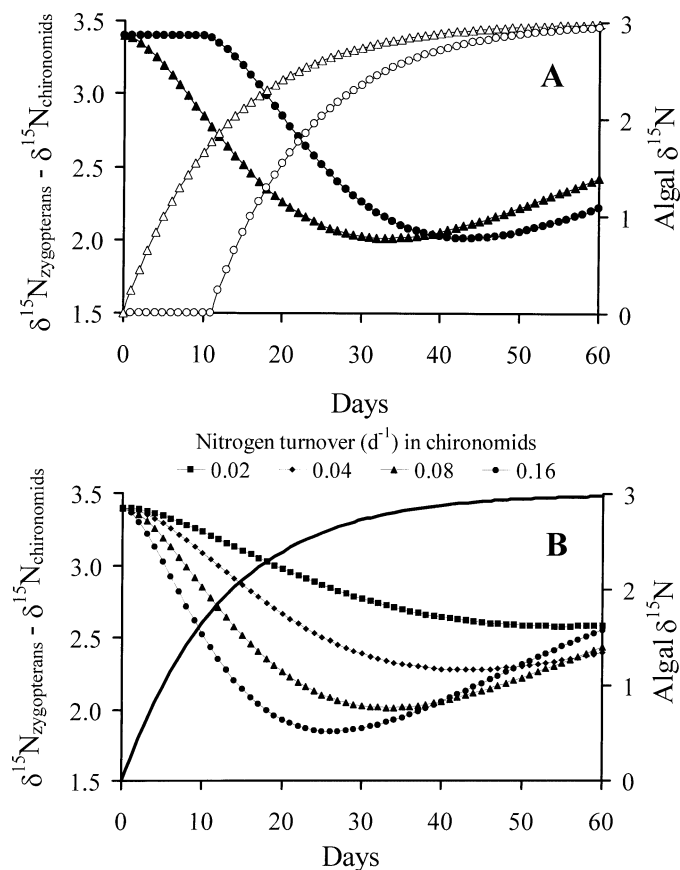


Fig. 3. (A) Simulated changes in  $\Delta\delta^{15}\text{N}$  between zygopteran larvae and their larval chironomid prey ( $\Delta\delta^{15}\text{N} = \delta^{15}\text{N}_{\text{zygopterans}} - \delta^{15}\text{N}_{\text{chironomids}}$ ) over time (black symbols) when a shift in  $\delta^{15}\text{N}$  of algae eaten by chironomids starts at different times (open symbols). The simulated foodwebs were identical, algae  $\rightarrow$  chironomid larvae  $\rightarrow$  zygopteran larvae, and included an enrichment of 3.4‰  $\delta^{15}\text{N}$  per trophic level. At day 25,  $\Delta\delta^{15}\text{N} = 2.1$  and 2.5, a difference that resulted only from the timing of the shift in algal  $\delta^{15}\text{N}$ . Eventually, these foodwebs would reach equilibrium and  $\Delta\delta^{15}\text{N}$  values would return to 3.4‰ in both foodwebs. (B) Simulations similar to those in part A, where the timing of a change in  $\delta^{15}\text{N}$  of algae is the same but the daily turnover rates of nitrogen in chironomid larvae differ between foodwebs. At day 25,  $\Delta\delta^{15}\text{N}$  values were 2.9, 2.5, 2.1, and 1.8, differences that resulted only from variations in chironomid turnover. Daily nitrogen turnover rates in chironomid larvae were 0.02, 0.04, 0.08, and 0.16. Nitrogen turnover in zygopteran larvae in all simulations was set to 0.03 per day.

opteran larvae and 0.02–0.16 per day for their larval chironomid prey. We used the commonly assumed enrichment 3.4‰  $\delta^{15}\text{N}$  per trophic level in all simulations (although in these simple simulations, this value only affects the y-intercept in Fig. 3).

In the first simulation (Fig. 3A), we compared two identical foodwebs that experienced different initiation dates for changing algal  $\delta^{15}\text{N}$ . Comparing foodwebs at day 25 yields  $\Delta\delta^{15}\text{N}$  between zygopterans and chironomid prey of 2.1 and 2.5‰. In the second simulation (Fig. 3B), we compared four foodwebs with the same initiation time of a shift in algal  $\delta^{15}\text{N}$  but with varying rates of nitrogen turnover for chironomids. Again, comparing foodwebs on day 25 gives  $\Delta\delta^{15}\text{N}$

values of 2.9, 2.5, 2.1, and 1.8‰. Thus, based on reasonable rates, it appears that differences in nitrogen-cycle dynamics could cause appreciable differences in  $\Delta\delta^{15}\text{N}$  between identical foodwebs. Even within the same foodweb, these effects could create difficulties when using  $\delta^{15}\text{N}$  values to delineate trophic pathways, especially for consumers that feed on multiple items with potentially varying turnover rates.

In conclusion, stable isotopes can provide a great deal of information about aquatic foodweb structure and function, but interpreting such data without independent evidence such as gut contents (or even with such evidence) can be problematic. Because macroinvertebrates often eat more than two foods,  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  can easily be interpreted to indicate trophic pathways that do not exist. Because  $\delta^{15}\text{N}$  fractionation varies widely with a number of factors, the usefulness of  $\delta^{15}\text{N}$  as an additional isotope for determining trophic pathways is inconsistent, and variations in  $\Delta\delta^{15}\text{N}$  may not always correspond to varying trophic levels or food-chain lengths (cf. Vander Zanden et al. 1999; Post et al. 2000). Particularly for macroinvertebrates in aquatic foodwebs, we urge that stable isotopes be supplemented with independent observations to avoid incorrect conclusions about trophic pathways, trophic levels, and food-chain lengths in different ecosystems.

*E. Andrew Hart and James R. Lovvorn*<sup>1</sup>

Department of Zoology  
University of Wyoming  
Laramie, Wyoming 82071

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<sup>1</sup> Corresponding author (lovvorn@uwyo.edu).

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