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Less means more: nutrient stress leads to higher $\delta^{15}N$ ratios in fish

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SUMMARY

1. Isotopic ratios of nitrogen are often used in food-web studies to determine trophic position (including food chain length) and food sources, with greater ratios of $^{15}N/^{14}N$ ($\delta^{15}N$) usually considered indicative of higher trophic position. However, fasting and starving animals may also show a progressive increase in $\delta^{15}N$ over time as they catabolise their own tissues.
2. To determine the importance of starvation, we conducted a 4-month laboratory experiment testing effects of starvation on body condition and isotope ratios in the muscle tissue of freshwater guppies (*Poecilia reticulata*). We also compared laboratory results and conclusions with analyses of body condition and isotope ratios in various small species of fish collected in four seasons from the Kansas River in north-eastern Kansas, U.S.A.
3. Fish starved in our laboratory experiment had significantly higher ^{15}N values and poorer body condition than those fed more regularly. The diverse group of fish species collected in summer (July) from the Kansas River had higher weight-to-length ratios and lower ^{15}N values than those retrieved in other seasons. Overall body condition resulting from reduced food consumption explained 44 and 53% of the variability in ^{15}N for field and laboratory fish, respectively.
4. These results are applicable to a wide variety of food-web research but are especially pertinent to studies of organisms that undergo large changes in life history, dormancy, extended fasts or periods of significant nutritional allocation to young.

Keywords: food chain length, nitrogen, starvation, trophic position

Introduction

An important metric of environmental health within ecosystems is food-web structure because it reflects species richness, lifestyle diversity and trophic interactions inherent among organisms. Recent studies have enabled us to investigate questions such as the following: What is the relative importance of ecosystem size, productivity, disturbance and habitat complexity in controlling food chain length (e.g. Post & Takimoto, 2007; Sabo, Finlay & Post, 2009; McHugh, McIntosh & Jellyman, 2010)? What factors control the relative importance to aquatic food webs of autochthonous vs. allochthonous carbon (e.g. Thorp *et al.*, 1998; Doucett *et al.*, 2007; Dudgeon *et al.*, 2010; Winemiller *et al.*, 2011)? And, what are the effects of anthropogenic impacts on food chain length and web complexity? Progress in answering these questions has greatly benefitted from use of stable isotope analysis (Peterson & Fry, 1987; Post, 2002; Roach, Thorp & Delong, 2009; Winemiller *et al.*, 2010, 2011).

Stable isotopes integrate food sources and reveal what an organism has generally assimilated over longer time scales compared with conclusions from shorter-term techniques, such as gut content and behavioural analyses. As organic matter is processed by cells, there is a tendency for selective retention of heavier isotopes and loss of lighter isotopes through chemical and physiological processes such as excretion or respiration. The change in relative abundance of heavy-to-light nitrogen isotopes ($\delta^{15}N = {}^{15}N/{}^{14}N$) can be used to estimate trophic position. The $\delta^{15}N$ of a consumer is typically

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considered to increase or become enriched by 3–4‰ relative to its diet (Deniro & Epstein, 1981).

Interpreting food sources and trophic position based on $\delta^{15}N$ may be misleading, however, because tissues of fasting and starving animals may also show a progressive increase in $\delta^{15}N$ as body mass decreases (Hobson, Alisauskas & Clark, 1993; Cherel *et al.*, 2005). This occurs because starving animals undergo catabolic processes, where they literally 'live on their own meat' (Waterlow, 1968). Indeed, a growing number of studies have proposed that the $\delta^{15}N$ ratio could be used as a general index of nutritional stress. This ^{15}N enrichment has been found in arctic ground squirrels (*Spermophilus parryii*) due to fasting (Ben-David *et al.*, 1999), Japanese quail chicks (*Coturnix japonica*) raised on different feeding regimes (Hobson *et al.*, 1993), hibernating American black bears (*Ursus americanus*) (Lohius, Harlow & Beck, 2007) and even humans suffering from anorexia nervosa (Mekota *et al.*, 2006).

Food-web studies using stable isotopes sometimes assume that seasonal effects or pronounced differences in food-web structure are due to changes in relative choices of prey type (Cherel *et al.*, 2007). However, these fluctuations could also result from starvation. If stable isotope ratios are indeed significantly affected by starvation or food availability, how do we interpret these results and differentiate them from an actual modification in choice of prey, and exactly how much are isotope values shifted?

We analysed the effects of nutritional stress (reduced food resources through starvation) on the nitrogen stable isotope composition of fish from the field (mixed riverine assemblage) and in a laboratory experiment with guppies (Poeciliidae, *Poecilia reticulata* Peters, 1859). We hypothesised that nutritionally stressed individuals would have significantly higher $\delta^{15}N$ ratios than more regularly fed fish (cf. Hobson *et al.*, 1993; Cherel *et al.*, 2005).

Methods

Laboratory feeding experiment

We examined the effects of feeding schedule on $\delta^{15}N$ of muscle tissue in 30 laboratory-raised guppies (*Poecilia reticulata*). Offspring from a laboratory-breeding population of these fish were fed *ad libitum* TetraFin Goldfish Flakes (42% crude protein, 8% crude fat, 2% crude fibre; http://www.tetra.net) for a period of 4 months on one of three schedules: daily, every 3 days or every 6 days. Fish were adults (sexually mature, 25–40 mm in length) and kept under a 12-h light/12-h dark cycle at *c.* 21 °C. Tanks were filtered continuously and cleaned weekly, to ensure no algae or debris accumulated. No experimental fish perished during the study.

Field study

We determined $\delta^{15}N$ of muscle tissue of field populations of small fish seined in 2013 from shoreline areas of the Kansas River near Lecompton, Kansas, U.S.A. (39.049664 N, 95.387214 W). Fish were sampled approximately every 3 months throughout the year (29 October, 1 February, 1 May and 29 July). During this period, we collected a total of 234 small fish for stable isotope analysis. These were a mixed assemblage of sand shiners (*Notropis stramineus*), red shiners (*Cyprinella lutrensis*), fathead minnows (*Pimephales promelas*), juvenile bluegill sunfish (*Lepomis macrochirus*), spotfin shiners (*Cyprinella spiloptera*) and mosquitofish (*Gambusia affinis*). The community composition varied somewhat among sample dates, but mosquitofish and spotfin shiners were typically most abundant, and thus, the overwhelming majority was used in the isotope analysis. All fish used in the analysis were identified to species, weighed and total length measured to determine body condition in terms of weight-to-length ratios. Only fish of sufficient size were used in the isotope analysis (25–50 mm in length).

Stable isotope analysis and trophic position

At the end of the laboratory experiment and following each field-sampling period, fish selected for isotopic determination were euthanised by ice water bath, body condition assessed and then stored in 75% ethanol. Fish were identified to species, and their body condition was determined from weight (g) measurements divided by the total length (mL) of the fish to give an overall ratio of body condition.

To determine isotope ratios, following the fish being stored for 1 week in ethanol, we removed a small plug of muscle from the body muscle tissue below the dorsal fin, rinsed it with deionized water and placed it in precombusted glass vials. Tissue samples were dried in an oven at 60°C for 48 hr and then ground into a fine, homogenised powder using a Wig-L-Bug® Mixer/Amalgamator (Rinn Corp./Crescent Dental Mfg. Co., Elgin, IL, U.S.A.). Subsamples (2.0–3.0 mg) were packaged into 4 × 6 mm tin capsules and held in desiccators until submitted for bulk-tissue stable isotope analysis.

We evaluated the nitrogen isotopic composition of fish muscle tissue and powdered laboratory flake food using

a ThermoFinnigan MAT 253 continuous flow system mass spectrometer (W. M. Keck Paleoenvironmental and Environmental Stable Isotope Laboratory, University of Kansas). The data for each sample included total N and $\delta^{15}N$ values. The $\delta^{15}N$ values were determined based on the relative difference in isotopic ratio between the samples and known standards, as represented by the following equation:

$$\delta X = \left(\left(\frac{R_{\text{sample}}}{R_{\text{standard}}}\right) - 1\right) \times 1000$$

where X is ^{15}N, the corresponding ratio is $R = {}^{15}N/{}^{14}N$, and atmospheric nitrogen was used as the N standard. All isotope ratios are given in per mil (‰).

The $\delta^{15}N$ signature of the muscle tissue collected from a fish typically integrates what it has eaten over the past 3 months (Maruyama *et al.*, 2001; Madigan *et al.*, 2012; Xia *et al.*, 2013). Therefore, the signature of fish collected in each of the different seasons in the field portion of the study represents an integration of the resources consumed since the previous season of collection.

Statistical analyses

We determined the effects of starvation and seasonality on body condition as well as nitrogen isotope ratios using ANOVA test (Minitab 14 statistical software; Minitab Inc., State College, PA, U.S.A.) with $\alpha = 0.05$. The relationship between nitrogen isotope ratios and body condition, associated with different seasons and feeding regimes, was determined by regression analysis and ANCOVA. The data were checked for normality and homogeneity of variances.

Results

The muscle tissues of guppies from our laboratory experiment fed every 6 days were significantly enriched in ^{15}N (Tukey's *post hoc* test; ANOVA, $F_{2,19} = 16.98$, $P < 0.0001$; Fig. 1a). Fish fed every 6 days also had a significantly lower body condition than the fish fed on more frequent schedules (Tukey's *post hoc* test; ANOVA, $F_{2,19} = 8.90$, $P < 0.002$; Fig. 1b).

In the field portion of the study, fish collected in July (=mid-summer) had a significantly lower $\delta^{15}N$ than fish analysed from the other three seasons (Tukey's *post hoc* test; ANOVA, $F_{3,71} = 62.02$, $P < 0.0001$; Fig. 2a), and the $\delta^{15}N$ of fish from other seasons did not differ from each other. Fish collected in July also had significantly higher weight-to-length ratios than those from other seasons (Tukey's *post hoc* test; ANOVA, $F_{3,71} = 58.55$, $P < 0.0001$; Fig. 2b).

Further analysis revealed that there was a significant overall relationship between $\delta^{15}N$ and body condition in both the laboratory ($R^2 = 53\%$, $F_{1,20} = 22.68$, $P < 0.0001$; Fig. 3) and field studies ($R^2 = 44\%$, $F_{1,73} = 57.63$, $P < 0.0001$; Fig. 4). The effect of starvation was seen both as an overall trend, when all of the data were combined into a scatterplot, as well as within specific treatment groups. When comparing treatment groups (i.e. different feeding schedule or season collected), there was no significant difference between the slopes (ANCOVA; Lab: $F_1 = 3.60$, $P = 0.094$; Field: $F_1 = 0.64$, $P = 0.437$; Figs 3 & 4).

Discussion

Stable isotope analysis is commonly used in food-web studies to determine food sources, trophic positions and

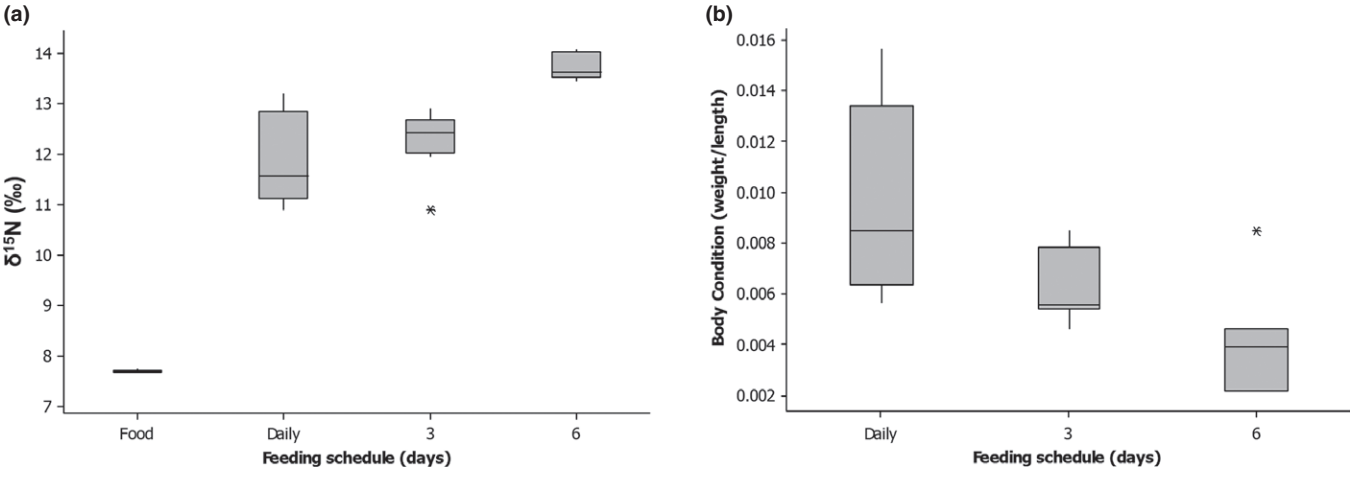

Fig. 1 (a) Guppy muscle tissue $\delta^{15}N$ signature (with TetraFin Goldfish Flakes) and (b) body condition (weight-to-length) interquartile range box plots of fish fed *ad libitum* one, 3 or 6 days. Outliers are indicated by asterisks.

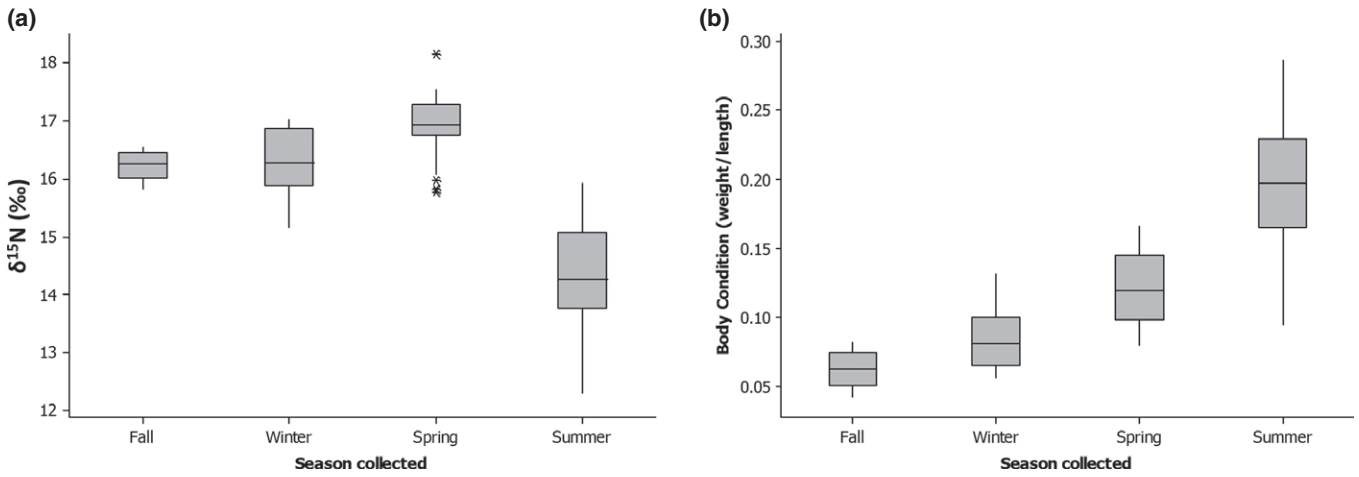

Fig. 2 Interquartile range box plots of: (a) $\delta^{15}N$ of muscle tissue and (b) body condition (weight-to-length) of small fish seined from shoreline areas of the Kansas River near Lecompton, Kansas, U.S.A., during four seasons of 2013. Outliers are indicated by asterisks.

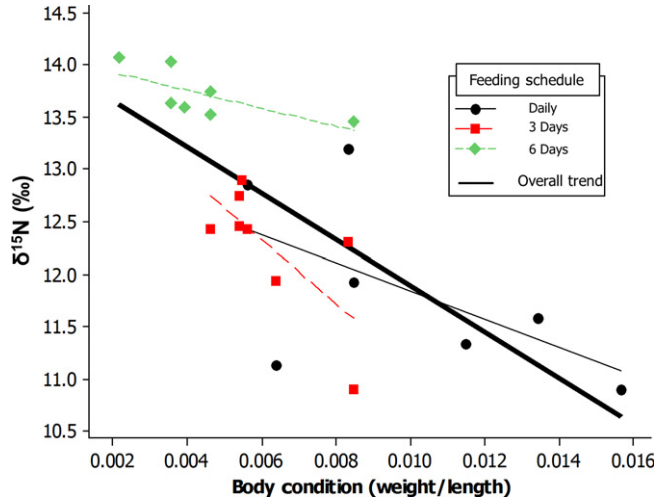

Fig. 3 Inverse relationship between $\delta^{15}N$ and body condition shown as an overall trend (R^2 = 53%) as well as within individual treatments of fish fed *ad libitum* one, 3 or 6 days.

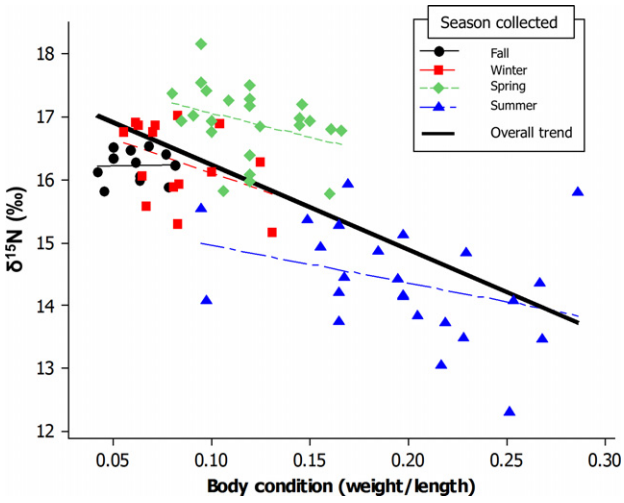

Fig. 4 Inverse relationship between $\delta^{15}N$ and body condition observed as an overall trend (R^2 = 44%) as well as within individual seasonal collections of small fish seined from shoreline areas of the Kansas River near Lecompton, Kansas, U.S.A., in 2013.

main interactions between organisms within an ecosystem. In determining these relationships, a number of assumptions are frequently made. Data are most commonly collected in the season of highest primary and secondary production when temperatures are relatively warm in all aquatic systems and river flow rates are moderate, with a common assumption that these periods are the most relevant ecologically and are sufficiently representative of food webs in other seasons. Another common convention in stable isotope ecology is that changes in an organism's isotope ratio are assumed to reflect changes in prey type, rather than the effects of other environmental factors, including starvation or altered isotope signatures of algae and other food sources (cf. Goering, Alexander & Haubenstock, 1990; Hobson *et al.*, 1993; Power, Guiguer & Barton, 2003; Woodland *et al.*, 2012).

Our results demonstrate that changes in nitrogen stable isotope ratios can indicate nutritional stress as well as differences in prey consumed. In our seasonal field study and laboratory feeding experiment, we found that *c.* 44 and 53% of variation in ^{15}N could be explained by a significant decrease in overall body condition resulting from reduced food consumption (as suggested by body length–weight ratios) in both field-collected and laboratory-raised fish, respectively (Figs 3 & 4). Our results with fish confirm similar studies of fasting and selective feeding in birds

(Hobson *et al.*, 1993; Kempster *et al.*, 2007). It is not clear, however, whether different levels of nutritional stress produce the same degree of nitrogen fractionation in all species.

The variability in body condition and $\delta^{15}N$ increased when fish had access to higher resource levels (Figs 1 & 2). In the field, this may have resulted from an increase in diet breadth. Some studies have suggested that omnivory becomes more common as food availability or ecosystem size increases (Williams & Martinez, 2004; Thompson & Townsend, 2005; Thompson *et al.*, 2007; Takimoto, Spiller & Post, 2008). In our laboratory experiment, increased variance under higher resource availability may have resulted from greater physiological generalisation, that is, differential allocation of resources to metabolic processes, organ systems or reproduction. Clearly, however, higher variance under increased resource levels indicates that $\delta^{15}N$ isotope ratios may be altered by additional interacting factors. More laboratory experiments are needed to test fully the assumptions and limitations inherent when interpreting isotope data, as they relate not only to nutritional stress but also to changes in behaviour and physiology associated with resource availability.

We recommend that investigators consider the possible effects of starvation when interpreting stable isotope data and evaluate the costs and benefits of determining body condition or nutritional stress level for their particular studies. Conclusions from our study may be particularly pertinent to studies of organisms that employ metabolic pathways that result in differing fractionation, undergo large changes in life history, experience extended periods of dormancy or are subjected to significant variation in food availability, the latter of which may include extended periods of fasting or care of young (including nursing) that monopolises the resources of the attending parent. By taking body condition into account, organisms may be more appropriately placed in a food web, thereby allowing better comparisons among studies and across taxa and ecosystems.

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