Oecologia (2006) 150:355–361
DOI 10.1007/s00442-006-0487-z

ECOPHYSIOLOGY

# Isotopic insight into host–endosymbiont relationships in Liolaemid lizards

**Shannon P. O'Grady · M. Denise Dearing**

Received: 8 November 2005 / Accepted: 16 June 2006 / Published online: 30 September 2006


**Abstract** Nitrogen isotopes have been widely used to investigate trophic levels in ecological systems. Isotopic enrichment of 2–5‰ occurs with trophic level increases in food webs. Host–parasite relationships deviate from traditional food webs in that parasites are minimally enriched relative to their hosts. Although this host–parasite enrichment pattern has been shown in multiple systems, few studies have used isotopic relationships to examine other potential symbioses. We examined the relationship between two gut-nematodes and their lizard hosts. One species, *Physaloptera retusa*, is a documented parasite in the stomach, whereas the relationship of the other species, *Parapharyngodon riojensis* (pinworms), to the host is putatively commensalistic or mutualistic. Based on the established trophic enrichments, we predicted that, relative to host tissue, parasitic nematodes would be minimally enriched (0–1‰), whereas pinworms, either as commensals or mutualists, would be significantly enriched by 2–5‰. We measured the $^{15}N$ values of food, digesta, gut tissue, and nematodes of eight lizard species in the family Liolaemidae. Parasitic worms were enriched 1±0.2‰ relative to host tissue, while the average enrichment value for pinworms relative to gut tissue was 6.7±0.2‰. The results support previous findings that isotopic fractionation in a host–parasite system is lower than traditional food webs. Additionally, the larger enrichment of pinworms relative to known parasites suggests that they are not parasitic and may be several trophic levels beyond the host.

**Keywords** Nematodes · Parasite · Symbioses · Diet

Communicated by David Post

S. P. O'Grady (✉)
Department of Pediatrics, Center for Pediatric Nutrition Research, P.O. Box 581289, Salt Lake City, UT 84158, USA
e-mail: shannon.ogrady@hsc.utah.edu

M. D. Dearing
Department of Biology, University of Utah,
257 South 1400 East, Salt Lake City, UT 84112, USA

## Introduction

Symbiotic relationships in the gastrointestinal (GI) tract include commensalisms, mutualisms, and parasitisms. Although symbiotic interactions are ubiquitous in nature (Herre et al. 1999), the characterization of these interactions is difficult (Bronstein 1994). The difficulty in characterizing symbioses arises primarily in attempting to measure the metabolic dependence of one organism on another, and secondarily in measuring the cost or benefit of this dependence.

Stable nitrogen isotopes have been widely used to track nutrient pathways and infer trophic relationships among organisms in complex food webs (Hobson 1993; Hobson and Welch 1992; Post 2002; Post et al. 2000). Isotopic analysis may facilitate the elucidation of host–symbiont relationships in the GI tract. The abundance of the heavier nitrogen isotope relative to the lighter nitrogen isotope, $^{15}N/^{14}N$, in a sample allows the inference of the trophic position of an organism. An enrichment of 3‰ is typical between the trophic levels for many ecological systems (DeNiro and Epstein 1981; Post 2002). However, a growing number of studies have reported variations around this number (but see Gannes et al. 1997; McCutchan et al. 2003; Robbins et al. 2005; Sponheimer et al. 2003), particularly with

respect to enrichments between hosts and parasites. The majority of nematode parasites are minimally enriched (0–1‰) with respect to the host tissue from which they are removed (Boag et al. 1998; Deudero et al. 2002; Iken et al. 2001; Olive et al. 2003; Pinnegar et al. 2001). The difference in isotopic enrichment in the host–parasite systems compared to other symbiotic interactions make stable isotopes a compelling tool to determine the nature of symbioses in the GI tract.

We used stable isotopes to determine if nematodes in the large intestine of herbivorous lizards were parasitic. Free-living nematodes or pinworms naturally occur in the hindgut of a wide variety of herbivorous amphibians and reptiles, and are of no apparent cost to the host (Dearing 1993; Iverson 1982; Nagy 1977; O'Grady et al. 2005; Pryor 2003; Pryor and Bjorndal 2003; Zimmerman and Tracy 1989). Pinworms only occur in the fermenting segments of the GI tract, are not attached to gut tissue, and are typically found in large numbers (>15,000: Iverson 1982). Roughly 40% of the total cellulase activity in the gut of herbivorous lizards has been attributed to pinworms, but it is unclear if the cellulase activity was attributable to pinworms or associated bacteria (Nagy 1977). A recent study on Bullfrog tadpoles (*Rana catesbeiana*) supports the hypothesis that pinworms are mutualists that enhance fermentation. Tadpoles with pinworms exhibited higher levels of volatile fatty acids and greater rates of fermentation, consequently leading to accelerated development and metamorphosis when compared to tadpoles without pinworms (Pryor and Bjorndal 2003). Although the presence of pinworms appears to be beneficial to the host, the majority of evidence to date has been anecdotal. Furthermore, pinworms in mammals have been exclusively characterized as parasites. Thus, if pinworms are commensals or mutualists, this would be the first nonparasitic relationship described in this nematode–host system.

Using the established isotopic relationships between consumers and their food sources combined with patterns of $^{15}N$ enrichment in host–parasite systems, we examined the role of pinworms in lizards of the family Liolaemidae. Liolaemid lizards have multiple nematode species throughout their GI tract (O'Grady et al. 2005). We compared the host–nematode enrichment of herbivores and pinworms with the enrichment of a known parasitic stomach nematode of insectivores and omnivores. We predicted that parasitic stomach nematodes would be minimally enriched (≤1‰) relative to the tissue of their hosts, and if pinworms were parasitic, they would show similar enrichments. Alternatively, if pinworms were not parasitic, they would be significantly enriched (2–5‰) relative to host tissue. Additionally, to control for the effect of variability in the wild diet (i.e., herbivory, omnivory, insectivory), we compared enrichment and pinworm size in a wild system with that of a captive herbivorous lizard on a control diet.

## Materials and methods

### Wild lizard species

Adult lizards were either noosed or caught by hand in a variety of habitats along the Andean cordillera in Argentina from 12 January 2003 to 13 February 2003 (latitudes: 26°S–43°S, longitudes: 66°W–70°W). Noosing occurred during the same time each day (1000–1400 hr) at sites ranging in altitude from 600 to 3,000 m. Species classifications were made based on both morphological data and mtDNA analysis (M. Morando and L. Avila, unpublished data). Lizards were euthanized with a pericardiac injection of Tiopental Sodico (Abbot). Stomach and large intestine contents were identified as either plant or arthropod using a 10× dissecting scope. The volume that was plant matter was recorded for both organs. Lizards were classified into diet categories based on the amount of plant matter contained in the stomach: herbivores ≥85%, omnivores 11–84%, insectivores ≤10%. Four herbivorous species, two omnivorous, and two insectivorous species were used in the study (Table 1).

### Captive lizard species

Lizards of the herbivorous species *Phymaturus antofagastensis* ($n$=4) were captured in Northwestern Argentina (26°51′S48, 66°44′W19) in late January 2003 and housed

**Table 1** δN15 of samples for each dietary group. If samples did not occur within a dietary group (i.e., stomach worms in herbivores), the cells boxes are labeled as not applicable (N/A). Values given are ±1 SD

| Lizard diet groups | Digesta | Stomach | Large intestine | Pinworms | Stomach worms |
|---|---|---|---|---|---|
| Captive herbivores[a] | 1.9 | 1.9±0.2 | 1.7±0.2 | 8.1±0.1 | N/A |
| Wild herbivores[b] | 4.2±0.9 | 6.3±0.6 | 5.8±0.7 | 12.5±0.7 | N/A |
| Wild insectivores[c] | 2.8±0.2 | 6.4±0.5 | 6.9±0.5 | N/A | 7.4±0.6 |
| Wild omnivores[d] | 5.6±1.4 | 6.2±0.3 | 5.8±0.5 | 12.9±0.7 | 7.1±0.2 |

[a] Phymaturus antofagastensis ($n$=4)

[b] Liolaemus buergeri ($n$=3), Liolaemus rothi ($n$=2), Phymaturus zapalensis ($n$=1)

[c] Liolaemus koslowskyi ($n$=3), Liolaemus darwinii ($n$=2)

[d] Liolaemus boulengeri ($n$=3), Liolaemus umbrifer ($n$=3)

at CRILAR-CONICET, Anillaco, Argentina, for use in a digestion trial. The lizards were maintained in a climate-controlled room at 22±3°C under a 13:11-h L:D cycle. Animals were housed individually in 30×20×10-cm plastic containers. The cage lids were modified with mosquito netting to allow in light and the cage bodies were covered with opaque paper to reduce the stress levels of the animals. Half of each cage was placed on a 28-cm span of CalorIQue heat tape and small rocks were placed on the bottom of each cage to facilitate heat dispersal. The heat tape was controlled with a thermostat and cage temperatures ranged from 34°C, in the unheated portion of the cage, to 44°C, in the heated portion of the cage. Cage temperatures were in the range recorded for this species in the field (S.P. O'Grady, unpublished data), and the lizards thermoregulated freely within this range. Additionally, each cage was provided with water and a large rock for basking. The temperature range of each cage, and the body temperature ($T_b$) and mass of each lizard were recorded daily. As part of a digestion study (results not presented in this paper), the animals were force-fed an artificial diet consisting of 20% yam mash, 20% ground high fiber rabbit chow (Harlan Teklad Global Diets, DE, USA), 20% calcium/phosphorus supplement (T-Rex Products Inc., CA, USA), and 40% water. Feces were removed daily to control for coprophagy. Captive lizards were maintained on the artificial diet for 3 weeks and were then euthanized with a pericardiac injection of Tiopental Sodico (Abbot).

### Nematode removal

All nematodes were removed from the digestive tracts of both wild-caught and captive lizards. For each lizard, the location (stomach vs. large intestine) of all nematodes was recorded. Nematode specimens from each organ were preserved in 70% EtOH for identification by Dr. Stephen Goldberg, Whittier College, California. Herbivores possessed pinworms, *Parapharyngodon riojensis*, insectivores possessed parasitic stomach nematodes, *Physaloptera retusa*, and omnivores possessed both species of nematodes.

### Nematode measurements

To compare the pinworm size between wild-caught and captive herbivores, nematodes were mounted onto slides and photographs were taken using a Leitz Laborlux K microscope with epi-fluorescence and a Nikon Coolpix camera. All photographs were taken at a magnification of 2.5×. To determine the length and width of pinworms, the dimensions were converted to pixels and measured with a micrometer.

### *Analysis of $^{15/14}N$ and $^{13/12}C$ isotope ratios*

Samples of gut tissue and nematodes were collected from the stomach and large intestine of each lizard and dried. Additionally, digesta was collected from the large intestine of each lizard and dried. Gut tissue and digesta samples were ground to a homogenous mixture; nematode samples were limited and, thus, were kept intact to obtain the necessary sample mass. All samples were weighed in tin capsules and stored in a desiccator until measurement. Isotope ratios were determined by a coupled system of an elemental analyzer (Costech ECS 4010) and a mass spectrometer (Finnigan MAT 252). Stable isotope abundance is expressed using the δ notation with $\delta X(‰)=(R_{sample}-R_{standard})/R_{standard}\times 1000$, where $X$ represents either $^{15}N$ or $^{13}C$. Depending on the isotope of interest, $R_{sample}$ and $R_{standard}$ represent either the $^{15}N/^{14}N$ or $^{13}C/^{12}C$ ratio of the sample and the standard, respectively. For $^{15}N$, atmospheric N served as the primary standard. For $^{13}C$, Peedee belemnite marine limestone was used as the primary standard. Yeast from the Stable Isotope Ratio Facility for Environmental Research (SIRFER) served as the internal standard for both $^{15}N$ and $^{13}C$. All samples were run in duplicate and averaged. Enrichment between two samples [e.g., worm (a) and gut tissue (b)] is expressed using the ε notation with $\varepsilon_{ab}=((\delta^{15}N_a+1000)/(\delta^{15}N_b+1000)-1)\times 1000$. The gut-nematode enrichment values refer to the region of the gut from which the nematode was removed (i.e., large intestine for pinworms and stomach for stomach worms).

### Statistical analysis

Species values in each study group (wild herbivores, wild omnivores, wild insectivores, and captive herbivores) were averaged. $\delta^{15}N$ and $\delta^{13}C$ values of tissue, digesta, and nematodes were compared between study groups using univariate analysis of variance (ANOVA). Enrichment in $\delta^{15}N$ and $\delta^{13}C$ (i.e., gut tissue-nematode, digesta-nematode) of the study groups were analyzed by ANOVA. If significant differences were found, the data were compared using a Tukey–Kramer HSD test. Differences in nematode size were analyzed using an unpaired *t*-test. The results were significant if $\alpha<0.05$.

## Results

### Lizards

Eight species of lizards were used in the analysis. All herbivores had pinworms, insectivores had stomach

worms, and omnivores had both pinworms and stomach worms (Table 1). No significant difference in $\delta^{15}N$ and $\delta^{13}C$ was detected between the stomach and large intestine tissue of herbivorous, omnivorous, and insectivorous wild lizards or between the stomach and large intestine tissue of captive lizards. Therefore, stomach and large intestine tissues of lizards will collectively be referred to as "gut tissue" for the remainder of this paper. The gut tissue of captive herbivores had a significantly lower $\delta^{15}N$ than wild herbivores, omnivores, or insectivores ($F_{3,38}$=33.8, $p$<0.0001; $p$=0.05, Tukey's HSD) (Table 1). Relative to the artificial diet, the gut tissue and large intestine contents of captive herbivores were enriched in $^{15}N$ by 1.4‰ and 1.5‰, respectively. Diet-tissue enrichments were not estimated for other dietary groups, since the actual diet was unknown. Digesta-tissue enrichments of wild herbivores, wild omnivores, and wild insectivores were not significantly different (1.9±0.4, 2.1±0.3, and 2.2±0.7‰, respectively).

## Nematodes

There was no significant difference in gut tissue-pinworm enrichment among captive herbivores, wild herbivores, and omnivores. Additionally, there was no significant difference in the enrichment of gut tissue-stomach worm between omnivores and insectivores (Fig. 1). There was a significant difference between tissue-pinworm enrichment and tissue-stomach worm enrichment (6.7±0.2‰ vs. 1±0.2‰, respectively; $F_{1,20}$=43.8, $p$<0.0001). This pattern was consistent among individual omnivores possessing both pinworms and stomach worms with tissue-nematode enrichments of 7.1±0.4‰ and 1.0±0.4‰, respectively (Fig. 1).

Digesta-pinworm enrichment was significantly lower in captive herbivores than wild herbivores or omnivores (6.2±0.1‰, 8.3±0.3‰, and 9.3±0.5‰, respectively; $F_{2,11}$=16.0, $p$<0.001; Fig. 2). Pinworms of captive herbivores had significantly lower $\delta^{15}N$ than pinworms of herbivores and omnivores (8.1±1‰, 11.9±0.7‰, and 12.9±1‰, respectively; $F_{2,11}$=6.5, $p$=0.01; Table 1).

## Nematode measurements

Pinworms of wild-caught lizard species were larger than pinworms from herbivorous species maintained in captivity. Pinworms from wild-caught species were both significantly longer (5.6±0.5 mm vs. 1.8±0.5 mm) and wider (1.0±0.1 mm vs. 0.4±0.1 mm) than pinworms in captive herbivores ($F_{1,16}$=29, $p$<0.0001; $F_{1,16}$=64.9, $p$<0.0001).

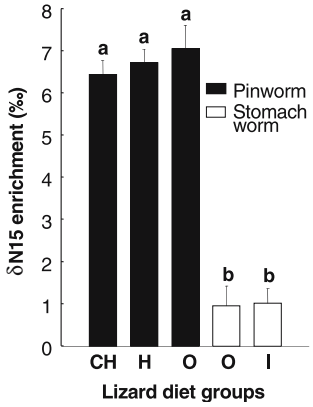


**Fig. 1** δN15 enrichment (Δ) between lizard gut tissues and nematodes of lizards that were captive herbivores (*CH*), wild herbivores (*H*), wild omnivores (*O*), and wild insectivores (*I*). *Filled bars* indicate pinworm-tissue enrichment and *open bars* indicate stomach worm-tissue enrichment. See Sect. 2 for explanation of the enrichment calculation. Groups with the same *subscript* are not significantly different

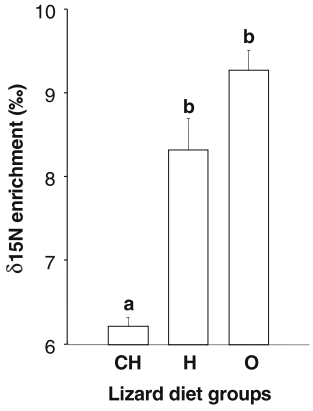


**Fig. 2** δN15 enrichment between lizard gut tissues and nematodes of lizards that were captive herbivores (*CH*), wild herbivores (*H*), and wild omnivores (*O*). See Sect. 2 for explanation of enrichment the calculation. Groups with the same *subscript* are not significantly different

## Discussion

We investigated the symbiotic relationship between GI nematodes and lizards of the family Liolaemidae by comparing $^{15}N$ values of this system to the established isotopic relationships between consumers and their food sources. The large differential between the $\delta^{15}N$ of pinworms and gut tissue across several species suggests that pinworms are not parasitic and may be commensals or mutualists feeding on gut microbes. In addition, pinworms of captive herbivores on a control diet were smaller and had dramatically lower $\delta^{15}N$, while pinworms of wild herbivores and wild omnivores did not

significantly differ in size or $\delta^{15}N$, despite variations in diet and nitrogen intake. In contrast, the large variation in the natural diets of wild omnivorous and herbivorous species did not directly affect pinworm size and $\delta^{15}N$. The artificial-diet-fed captive herbivores may have indirectly affected pinworms by altering the composition and/or stability of the microbial community. Finally, our results confirm previous findings that endosymbiotic parasites are minimally enriched with respect to host gut tissue.

## Stomach worm enrichment

Minimal host–parasite enrichment (1±0.2‰) was consistent in both insectivorous and omnivorous lizard species, indicating that the enrichment factor of stomach parasites is independent of diet and is likely due to the metabolic dependency of the parasite on the host. Endosymbiotic parasites are generally minimally enriched or are depleted relative to their host (Boag et al. 1998; Deudero et al. 2002; Iken et al. 2001; Olive et al. 2003; Pinnegar et al. 2001). Minimal enrichment of parasites is partially caused by direct absorption and incorporation of amino acids from the host. In the presence of excess amino acids, parasites down-regulate transamination and deamination pathways (Kohler and Voigt 1988). Many essential amino acids are depleted in $^{15}N$; this and the lack of amino acid processing may lead to the isotopic relationship between parasites and their hosts (Deudero et al. 2002; Hare et al. 1991). Thus, host diet and nutrition should have a minimal effect on parasite fitness because the amino acid profile of the host should not change extensively, unless the host is in a prolonged state of fasting/starvation. Additionally, the reutilization of ammonia by the parasite via reversal of the glutamate dehydrogenase pathway may further reduce enrichment between the host and parasite (Barrett 1981; Deudero et al. 2002; Olive et al. 2003).

## Pinworm enrichment

If pinworms were parasitic, we expected minimal enrichment relative to the host lizard, comparable to the observed enrichment factor of stomach parasites and lizard hosts. The enrichment factor between pinworms and lizards was much higher (6.72±0.2‰) than expected if they were parasitic, and suggests that pinworms do not feed on host tissue or blood. Additionally, the $\delta^{15}N$ enrichment between gut tissue and pinworms was consistent among captive herbivores on an artificial diet, wild-caught herbivores, and wild-caught omnivores. The consistency of the tissue-pinworm enrichment between the species studied ($n$=6) and across dietary groupings (i.e., herbivorous and omnivorous diets) indicates that this pattern is unaffected by large variations in diet composition. Furthermore, the patterns of enrichment for parasitic stomach nematodes and their hosts and the large enrichment between host tissue and pinworms were consistent within individuals. For example, all omnivorous lizards possessed both types of nematodes within their GI tract and the enrichment patterns between host tissue–parasitic worm and host tissue–pinworm were similar to that observed in individuals hosting a single species of nematode. Pinworms may consume nutrients from digesta, microbes within the host gut, and/or byproducts of microbe digestion. Digesta-pinworm enrichment was high in the captive system (6.2±0.1‰) and even higher in the wild herbivore and omnivore systems (8.3±0.4‰ and 9.3±0.2‰, respectively). Enrichment factors of this scope suggest that pinworms are unlikely to be consuming nutrients directly from digesta and may be a few trophic levels beyond the digesta. Furthermore, larger enrichment values in the wild system may be a result of greater dietary diversity and/or more complex microbial communities. Pinworms may specialize on microbes within the host gut, and/or byproducts of microbe digestion. Bacteria cultured on individual amino acid substrates show enrichment factors ranging from $<-12$ to >22‰ (Macko and Estep 1983). The large pinworm enrichment factors in this study, 6–9‰, could be an indication that pinworms are consuming either a single microbe species, specializing on a specific amino acid or a predatory symbiont consuming bacteria.

## Captive–wild comparison

Despite consistent tissue-pinworm enrichment in wild and captive systems, substantial variation existed in $\delta^{15}N$ values for tissue, digesta, and pinworms. Low enrichment in the captive system is suggestive of poor nutrition, although conclusions would be premature, given the small temporal window of data collection for wild species. In addition to variations in $\delta^{15}N$, the pinworm size differed significantly between captive species and wild species. Pinworms in captive herbivores appeared to be negatively affected by the switch to an artificial diet, as evidenced by their smaller size. Such differences lead to interesting questions about nutritive flow and fractionation between diets, but the lack of data on the diet of the wild species examined precludes the drawing of firm conclusions. Given that pinworm enrichment is consistent across lizard species and diet type, a study of a single species combining dietary data

from the field with laboratory manipulations could appropriately address this issue. Field studies of isotopes would provide ecologically relevant enrichment patterns, while laboratory manipulation of diet would yield precise information about fractionation patterns and nutrient flow in the system. Focusing exclusively on either field or lab approaches may lead to an inaccurate evaluation of the pinworm–lizard symbioses.

## Future directions

The results of this study did not support the hypothesis that pinworms are parasites. Additional research is necessary to resolve the large enrichment factor between pinworms and their lizard hosts. A variety of physiological processes are likely to affect the nitrogen signature of both lizard hosts and pinworms. To elucidate this relationship, it is necessary to determine the flow of nutrients from host to endosymbiont, and potentially back to the host. Isotope analysis of individual amino acids would provide a precise and controlled method for nutrient tracking in this system. By tracking the enrichment of a specific amino acid, we could determine if pinworms were consuming microbes in the hindgut and, potentially, contributing to host nutrition.

**Acknowledgments** S.P.O'G. was supported by a teaching assistantship from the University of Utah, USA, and this study was funded by a grant in aid of research awarded to S.P.O'G. from the Society for Integrative and Comparative Biology. M.D.D. was supported by NSF IBN 0236402. The authors wish to thank Mariana Morando and Luciano Avilla for their assistance in obtaining samples and Matt Sponheimer, Kent Hatch, Adam West, Naomi Levin, Ben Passey, David Post, and the anonymous reviewers for their critical and helpful comments. Additionally, much is owed to Thure Cerling for the use of his laboratory and Joseph Griggs for the sample preparation. Trapping permits were provided by the fauna authorities of the Catamarca, San Juan, Chubut, and Neuquen provinces of Argentina. This experiment was conducted in accordance with the current laws of the United States of America and Argentina.

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