USING NITROGEN-15 TO EXAMINE PROTEIN SOURCES IN HUMMINGBIRD DIETS

Uso de nitrógeno-15 para examinar las fuentes de proteína en las dietas de los colibríes

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ABSTRACT

Hummingbirds rely on the sugars in nectar to meet their high metabolic requirements, but most nectars are extremely low in nitrogen. As a result, the birds must also consume arthropods to meet their protein requirements. In many hummingbird species, males use nectar resources differently from females. I hypothesized that the sexes might also differ in their use of arthropods, because breeding females have higher protein requirements. I used $\delta^{15}N$ isotopes in feathers and blood to demonstrate that females feed at higher trophic levels than males and adults at higher levels than juveniles, respectively. Females captured during the breeding season were also feeding at higher trophic levels than those captured outside of the breeding season, though the sample sizes were small. I also found a slight but unanticipated increase in $\delta^{15}N$ values in feathers with elevation in one species.

Key words: Arthropods, tropical cloud forest, hummingbird, nitrogen isotopes.

RESUMEN

Los colibríes dependen de los azúcares del néctar para suplir su elevada demanda metabólica, pero la mayoría de los néctares son extremadamente pobres en nitrógeno. Como consecuencia, estas aves deben consumir también artrópodos para satisfacer sus necesidades proteicas. En muchas especies de colibríes, los machos y las hembras utilizan los recursos florales en forma diferente. Propuse que los sexos también podrían diferir en el consumo de artrópodos, porque las hembras tienen mayores demandas de nitrógeno durante la época reproductiva. Empleé isótopos $\delta^{15}N$ de plumas y sangre para demostrar que las hembras se alimentan en niveles tróficos más altos que los machos y que los adultos lo hacen en niveles más altos que los juveniles. Las hembras capturadas durante la temporada reproductiva también se alimentaron en niveles tróficos más altos que las capturadas fuera de la época de cría, aunque el tamaño de las muestras fue pequeño. También encontré un leve pero inesperado aumento en los valores de $\delta^{15}N$ en las plumas con elevación en una especie.

Palabras clave: Artrópodos, bosque de niebla tropical, colibrí, isótopos de nitrógeno.

INTRODUCTION

Hummingbirds are best known as nectarivores, but also supplement their diet with arthropods and probably some fruits. They rely on sugar-rich nectars, which are extremely low in nitrogen, to meet their high metabolic requirements. These birds have a suite of adaptations to their nitrogen-poor diets, including lowered nitrogen demands (McWhorter et al. 2003, Tsahar et al. 2005) and specialized bacteria in their guts to recycle nitrogen (Preest et al. 2003). Such adaptations to conserve nitrogen imply an important role for protein sources in hummingbird

diets. Meeting protein needs may figure in their behavioral and physiological adaptations more than has been recognized. Indeed, the availability of arthropods may determine the timing of breeding in dry tropical forests (Poulin & Lefebvre 1997).

Compared to females, male hummingbirds typically spend more time defending their territories (Chai et al. 1996, Temeles et al. 2002, Van Dooren et al. 2004), less time tending the nest (Baltosser 1996), and do not feed the young (Stiles 1995). The sexes may also employ different foraging strategies to reduce intraspecific competition (Temeles et al. 2002). Although these gender differences are well-studied in relation to nectar resources, there is very little information on arthropod foraging. One study in the Colombian Andes found female hummingbirds are more likely to have spiders in their guts than males (Rico-G. 2005). Female birds of other species have been found to have higher protein requirements than males (Moore et al. 2000) and metabolize more protein (Durant et al. 2000) during egg production. Thus, I hypothesized that female hummingbirds might differ from males in their selection of arthropods, because of their likely higher protein requirements for breeding. Furthermore, I hypothesized that juveniles' diet might be more similar to females', because they also face high nitrogen demands for growth.

This study compares the arthropod component of diet among three hummingbird species common in the Eastern Andes, and between genders, age classes, and seasons in the two of these species that are sexually dimorphic. *Adelomyia melanogenys* (Speckled Hummingbird) is a small, monomorphic (though males are usually larger than females), short billed generalist (Altshuler 2006). The other two species are strongly dimorphic, so their sexes can be readily distinguished in the field. *Heliodoxa leadbeateri* (Violet-fronted Brilliant) is stocky and aggressively territorial (Altshuler, 2006), and shows marked sexual dimorphism not only in plumage, but also in the males' greater size and slightly shorter, straighter bills. In *Coeligena torquata* (Collared Inca), the sexes are also readily distinguished by plumage. Males are slightly larger but on average, have shorter bills. Their behavior has not been classified, but a Peruvian congener was described as a "filcher" (Altshuler 2006). Filchers steal nectar from territories defended by other birds. In Colombia, males sometimes defend territories based on preferred flowers if these are in sufficiently dense aggregations; at other times they may trapline or, more rarely, filch (F. G. Stiles, pers. comm.). Birds that employ stealing strategies regularly get into to aggressive interactions (unlike trap-lining hummingbirds which make repeated visits to a series of plants), but almost never as aggressors.

It is easy to see hummingbirds catch arthropods on occasion, but difficult to gather systematic data for prolonged periods. In fact, there are no published data sets that quantify gender differences in arthropod foraging behavior. Stiles (1995) did not demonstrate any sex-specific arthropod foraging in a long-term study at a lowland rainforest site in Costa Rica. Furthermore, nectar leaves almost no trace in the digestive tract, so gut analyses of hummingbirds cannot reveal information on nectar use, and foraging observations are biased toward easily-observed flower visits (Stiles 1995). Given this difficulty in evaluating protein sources via direct observations, isotopic analysis can provide an alternative (Post 2002).

Nitrogen enters ecosystems primarily from the atmosphere, where the stable isotope $\delta^{15}N$ is 0.366% of total N. Once incorporated into biological systems, metabolic pathways favor ^{15}N during each step of protein synthesis. Therefore, top predators have appreciably higher ^{15}N:^{14}N ratios than autotrophs. In general, the change in $\delta^{15}N$ between trophic levels is 3.4±1.3‰ (Post 2002). Thus, $\delta^{15}N$ (defined as $[(R_{SAMPLE} - R_{STANDARD})/ R_{STANDARD}]*1000$, where R_{SAMPLE} is the measured ratio of ^{15}N:^{14}N in the sample, and $R_{STANDARD}$ is the known ratio of ^{15}N:^{14}N in air) can be used to evaluate the relative importance of different foods in an animal's diet because higher $\delta^{15}N$ values imply feeding at higher trophic levels. For instance, this method has successfully distinguished bats that feed primarily on fruit from bats that consume more arthropods (Herrera et al. 2001), and revealed that tropical passerines rely more heavily on protein from fruits than protein from insects when fruits are most abundant (Herrera et al. 2005).

In this study, I rely on an important difference between the way dietary nitrogen is incorporated in feathers, and the way it is incorporated into whole blood. Feathers are inert material, their composition permanently fixed at growth. Blood, on the other hand, reflects the animal's diet in the 5-10 days before sampling. In addition, the $\delta^{15}N$ signatures differ slightly between feathers and blood simply because of the metabolic pathways involved in the production of each tissue. Keratin in feathers has a slightly higher $\delta^{15}N$ signature than blood even in laboratory experiments where birds are fed a constant diet (Hobson & Bairlein 2003).

Because they require nitrogen to supplement their largely nectar-based diet, nearly all hummingbirds forage for arthropods in one fashion or another, primarily by hover-gleaning, or by hawking (Stiles 1995). The prey taken by hover-gleaning might include small spiders and prey stored in spider webs, whereas hawking behaviors will only capture flies. Because spiders feed at a higher trophic level than most flies, their tissues are expected to contain a higher proportion of $\delta^{15}N$. That higher proportion should be reflected in the tissues of hummingbirds that consume a large fraction of spiders, as was observed in female hummingbirds in Colombia (Rico-G. 2005). I determined the $\delta^{15}N$ ratio and total nitrogen content of flies and small spiders, and then compared that to the $\delta^{15}N$ in hummingbird feathers.

MATERIALS AND METHODS

STUDY AREA.- Sangay National Park, on the western edge of the Amazon basin, extends from the peaks of some of Ecuador's tallest volcanoes into the lowlands of the tropical rainforest. It represents some of the most intact contiguous forest remaining on the eastern slope of the Andes in Ecuador. My research sites were along the new road which bisects the park or the backcountry trail to the Sardinayacu Lakes (Fig. 1). The precise locations were Purshi, 2500 m (2°12'S, 78°23'W); Lago, 1750 m (2°04'S, 78°13'W); Primero, 1550 m (2°05'S, 78°11'W; and Nueva, 1350 m (2°06'S, 78°09'W). At each site, I cleared net lanes opportunistically, near hummingbird flowers or along small ridges. On each visit, I captured birds in mist nets (12 m, 4 shelf, 32 mm mesh). Each sampling day, I ran 10-15 nets for approximately 8 hours, beginning at sunrise. Because capture rates dropped precipitously even on the second day, I tried to net no more than three days per site per month.

FIELD METHODS.- I observed several species of hummingbirds visiting tiny spider webs on a rock wall near my field site, so I collected arthropods from the webs and stored them in 99% ethanol for isotopic analysis. In most cases, it was easy to find and collect the spider. I occasionally found remnants of tiny flies in the webs, but never sufficient to sample. I collected live flies and microhymenopterans from near plants where I saw birds perched. I also collected fruit (blackberry) and pollen from *Fuchsia* sp. I collected only small arthropods, and it was necessary to homogenize all the samples to achieve sufficient mass for the elemental analyzer.

Between July and October, 2004, in April 2005, and between July and October, 2006, I captured 22 *A. melanogenys*, 28 *C. torquata*, and 24 *H. leadbeateri* and collected two retrices and ~ 75uL of blood from a clipped claw from each bird (IACUC protocol A126-06-04). Juveniles were identifiable by minute striations along the length of their bills. Also, as with other species of *Heliodoxa* (cf. Ridgway 1911), juveniles of *leadbeateri* have prominent buffy to rufous borders to the malar stripe, which are visible even through binoculars. I stored the retrices in envelopes at ambient temperature and preserved the blood in 1.5 mL tubes with 99% ethanol until analysis.

I attempted to make direct observations of arthropod foraging by hummingbirds, but met with limited success. I was only able to record foraging activities by *H. leadbeateri* and *C. torquata* 12 and 7 times, respectively. For half of the *H. leadbeateri* observations, I was unable to determine the bird's sex.

LABORATORY AND STATISTICAL METHODS-. I cleaned the feathers of surface oils using 2:1 chloroform: methanol solution, and then dried them overnight in a fume hood. I then cut 1.00 mg

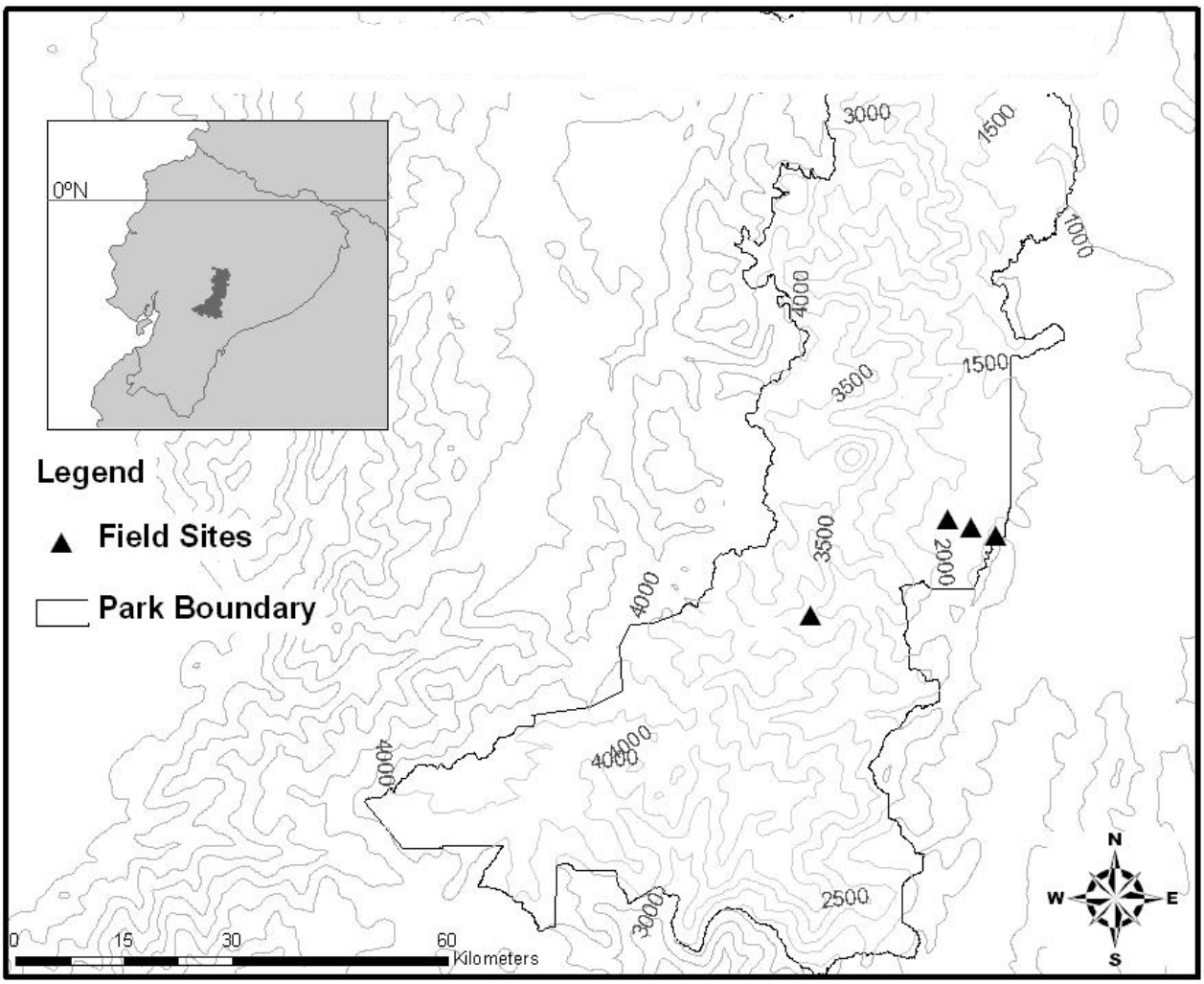

Figure 1. Study sites in Sangay National Park, on the eastern slope of the Andes in Ecuador.

samples from the feather vane and packaged them in tin cups. I freeze-dried the blood and arthropods in a lyophilizer overnight, and then weighed out 0.35 mg samples. The samples were run in the Duke Environmental Stable Isotope Laboratory on a Carlo Erba Elemental Analyzer Mass Spectrometer with precision of duplicate samples ± 0.11‰. Standards were composed of international reference materials IAEA N_2, and Duke University internal standards, Duke Cellulose, Duke Sucrose, Duke Urea, and Costech acetanilide. The laboratory reports precision as generally ± 0.2‰ relative to external standards and a little better relative to internal standards.

For data analysis, I used parametric approaches and R software. To compare among sub-specific groups, I used a two-way fixed-effects analysis of variance with interaction, which assumes a normal distribution. The data violated the rule of thumb that no group should have a standard deviation more than double that of another (juvenile *A. melanogenys* and *C. torquata*), but since the sample sizes were so small, I considered this acceptable. I used paired t-tests (with pooled variance) to compare blood to feather values, and to compare female blood values between seasons. Since I had strong predictions about both of these relationships (that feathers and breeding season blood samples would be higher), I used a one-tailed hypothesis test. Finally, I also used linear models to examine the relationship between blood and feathers, and the effect of elevation on $\delta^{15}N$ values in feathers.

RESULTS

PROTEIN SOURCES.- The spiders I collected contained both a higher concentration of nitrogen and a higher $\delta^{15}N$ than flies (Table 1).

Table 1. Spiders had both higher percent nitrogen composition and a higher $\delta^{15}N$ value than other potential sources of hummingbird dietary protein at 2500 m elevation. Because samples were small, and material was homogenized for analysis, error estimates are unavailable.

	N	$\delta^{15}N$
Spider	14.6 %	7.4‰
Flies	11.0%	3.5‰
Fuchsia sp. Fruit	0.9%	4.0‰
Fuchsia sp. Pollen (n = 2)	3.7±1.9%	-5.1±4.4‰

EFFECTS OF SPECIES, AGE AND GENDER.- $\delta^{15}N$ of tail feathers varied by species, age class, and gender (for the species which I was able to sex in the field). I compared the effects of species and age class on feather $\delta^{15}N$ for all three species. An analysis of variance yielded a significant main effect for both species, $F(1,74) = 12.7$, $p<0.001$, and age class, $F(1,74) = 6.2$, $p = 0.015$, such that $\delta^{15}N$ was significantly lower for juveniles (mean = 6.34‰, SD = 2.10) than for adults (mean = 7.53‰, SD = 1.24; Table 2). I was unable to compare sexes in the monomorphic *A. melanogenys*, but feathers from female *C. torquata* showed significantly higher $\delta^{15}N$ levels (one tailed $t_{20}=1.73$, $p=0.05$) than males. There was weaker evidence for a difference between sexes in *H. leadbeateri* (one tailed $t_{16}=1.63$, $p=0.06$).

Table 2. ANOVA results showing primary effects indicate that both species and age class had a significant effect on $\delta^{15}N$ values of hummingbird feathers.

Effects	SS	df	MS	F	P
Species	42.89	2	21.45	12.69	<0.001
Age class	10.44	2	10.44	6.18	0.015
Interaction	3.76	2	1.88	1.16	0.334
Residuals	118.8	70	1.69		

Previous studies of trophic position have examined $\delta^{15}N$ in blood rather than in feathers (Herrera et al. 2003, Herrera et al. 2006), so I examined the relationship between $\delta^{15}N$ in blood and feathers in my data. Because feathers have slightly higher $\delta^{15}N$ values, even in birds fed a consistent diet, I expected my feather $\delta^{15}N$ values to be slightly higher than the blood $\delta^{15}N$ values from previous research. Although I was unable to collect and analyze blood from each individual used in the feather study, I had both blood and feather data for a total of 30 hummingbirds of a variety of species. In these birds, the mean $\delta^{15}N$ in blood was 2.12‰ lower than that of feathers (paired $t_{30}=-10.23$, $p<0.001$). This is comparable with laboratory values of a 1.7‰ difference between blood and feathers in *Sylvia borin* (Garden Warblers; Hobson & Bairlein 2003). Regression analysis comparing blood and feathers from given birds showed a highly significant relationship ($F_{1,29}=62.0$, $p=.0004$) (Fig. 2). Blood samples from the present study ranged from 5.03‰ for a juvenile *A. melanogenys* to 8.24‰ for an adult *C. torquata*. However, values of $\delta^{15}N$ in feathers and blood were generally in the same range as those of spiders.

EFFECT OF SEASON.- Because samples sizes of blood were so small, I used feathers for most comparisons, and blood $\delta^{15}N$ only to compare females captured during breeding and non-breeding seasons. This comparison could only be done with blood, because it required a tissue that integrated values over a short period of time, and offered current values. Because feathers are inert, they capture information only from the period during which they were grown. Juvenile hummingbirds were common in the nets during October and November. Blood samples from female *H. leadbeateri* had lower $\delta^{15}N$ during the months that no juveniles were sighted (5.68 ± 0.26‰) than when there were juveniles present (6.77 ± 0.08‰). The difference was significant (one tailed $t_9=3.44$, $p=0.003$ $n_{breeding} = 5$, $n_{non\text{-}breeding}= 4$). Molt was most common in October, which was immediately after breeding.

EFFECT OF ELEVATION.- There was a significant, though noisy effect of elevation on $\delta^{15}N$ *in A. melanogenys* ($F_{1,09}=7.716$, $p=0.01$) (Fig. 3).

DISCUSSION

There are clearly some differences in the diet of the

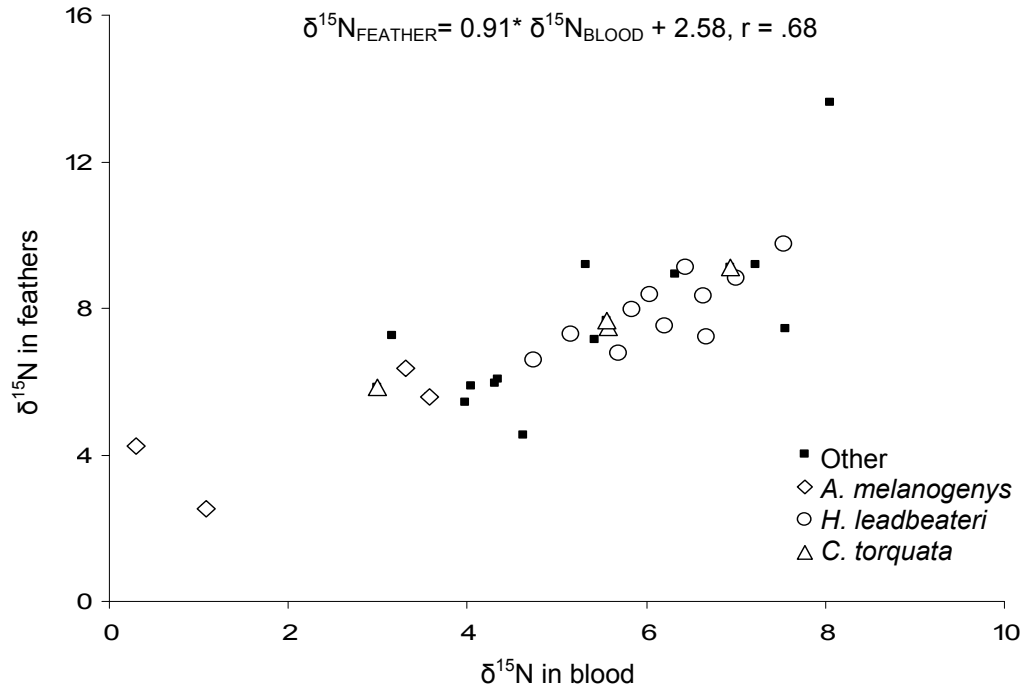

Figure 2. Feather $\delta^{15}N$ values of Andean hummingbirds averaged 2.12‰ higher than blood. "Other" designates hummingbird values from species in addition to the focal species.

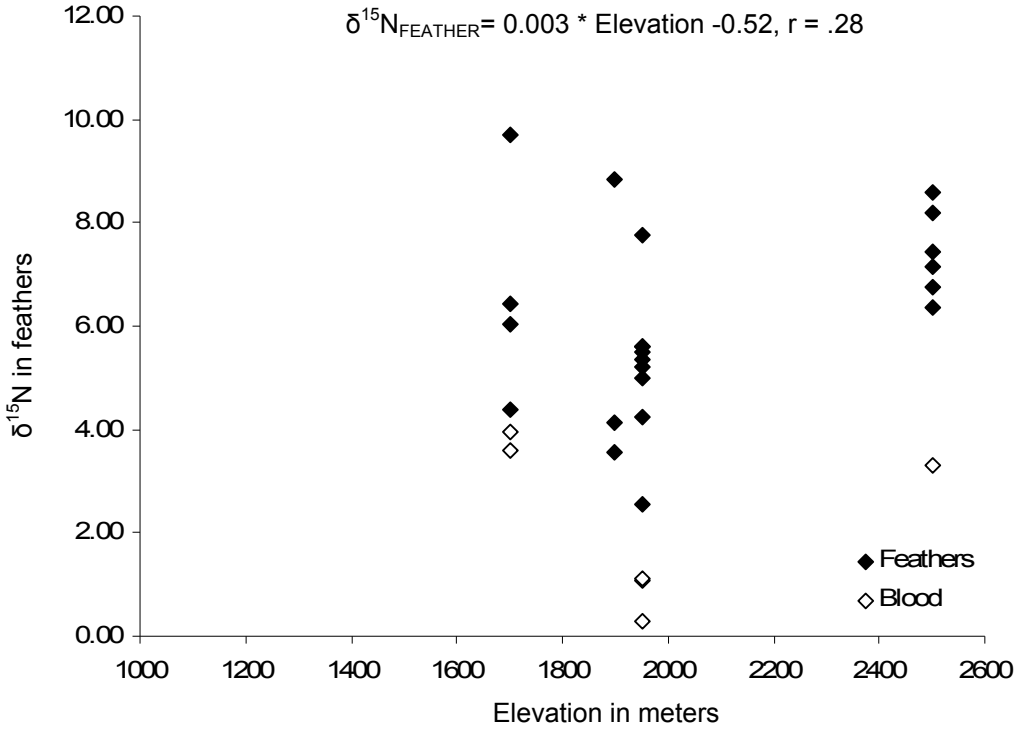

Figure 3. $\delta^{15}N$ in *A. melanogenys* feathers bore a slight but significant relationship to elevation ($F_{1,20}$=7.716, p=0.01). Blood values shown for comparison. The possibility of such an effect should be considered in future studies.

smaller, lighter, shorter-billed *A. melanogenys* compared to *H. leadbeateri* and *C. torquata*. *A. melanogenys* individuals are probably using a wider variety of protein sources, perhaps incorporating some nitrogen from fruit. The differences between the two dimorphic species were minimal, though the behavior of *H. leadbeateri* might have led us to expect lower $\delta^{15}N$ values since it is the more territorial of the two species, which is correlated with higher burst power in flight (Altshuler 2006). Higher burst power generally entails less ability to hover (Chai et al. 1996), so we might have expected that *H leadbeateri* would prefer flying prey to that obtained by raiding webs, thus consuming fewer spiders and having lower $\delta^{15}N$. This analysis failed to detect such a difference.

The results suggest that female hummingbirds feed at a higher trophic level than males (Table 3), which is consistent with a recent behavioral study of Colombian hummingbirds that found females relying more heavily on spiders than males of the same species (Rico-G. 2005). The differences in that study were especially marked in *Coeligena helianthea*, a congener of *C. torquata*, one of the species included in my study. Isotopic analysis deals only with assimilated protein (Herrera et al. 2005) in tissues, so that a higher $\delta^{15}N$ does not imply more total protein in the diet. However, spiders are both richer in protein and have higher $\delta^{15}N$ than flies, so female hummingbirds are likely assimilating more protein (Table 3). Moreover, previous studies examining isotopic variation have only discriminated among species of different guilds (Herrera et al. 2003). The present work demonstrates that ^{15}N can also be used to discriminate within the rather narrow guild of nectarivores, and even further, within species.

Sexes may differ in their prey selection because the trade-offs of consuming volant vs. non-volant arthropods play out differently for male and female hummingbirds. Males generally have narrower wings, even in species that have almost no sexual dimorphism (Stiles 1995 and pers. comm.). Their narrower wings are thought to give them greater maneuverability and more powerful flight, which is useful in territory or mate defense, but makes hovering more difficult and more energetically demanding (Chai et al. 1996). Hence males may be eating more volant arthropods because they are less costly to catch. Aerial foraging strategies may also be less disruptive to maintaining vigilance of their territories. On the other hand, females have broader wings and they also face higher nitrogen demands. Therefore, hovering and surface gleaning (and thereby taking more spiders) might be more energetically efficient for females than males.

The higher $\delta^{15}N$ values in the blood of females during months when juveniles were also present indicates a breeding season shift in arthropod diet, possibly in response to the high nitrogen demands of reproducing. This intra-annual difference in diet underscores the importance of protein sources to the hummingbirds. The results are consistent with the hypothesis that demands of protein synthesis during breeding can result in generalized sex differences in avian resource use.

Finally, I hypothesized that juveniles would have $\delta^{15}N$ levels comparable to those of females because they also face high protein requirements for their growth. Instead, they generally showed lower values of $\delta^{15}N$ than adults of either sex. This might be because juveniles lack the skills or stamina to forage on the same prey as adults, so they forage for different prey on different substrates.

The results of my research could be confounded by three things: other sources of nitrogen, metabolic or physiological differences between genders, or other unexplained effects. The role of other sources of nutrients, such as pollen and fruit, is not likely to be

Table 3. Female hummingbirds tended to have higher $\delta^{15}N$ values in their feathers. The $\delta^{15}N$ values were lower in juveniles. Values are Mean ± 1 SD, (sample size).

Species	Adults	Males	Females	Juveniles
A. melanogenys	6.53 ±1.2‰ (16)	N/A	N/A	5.03±2.12 ‰ (7)
H. leadbeateri	7.95±0.96‰ (21)	7.39±0.99‰ (9)	8.09±0.97‰ (12)	7.22±0.96 ‰ (4)
C. torquata	7.84±0.94‰ (22)	7.55±1.04‰ (12)	8.24±0.82‰ (10)	7.55±1.94 ‰ (5)

great in the diet of any hummingbird species. Pollen is probably too well protected to be digestible by hummingbirds' rather weak digestive system (van Tets & Nicolson 2000), and fruit has generally such a low total nitrogen content that it is incapable of contributing very much protein to a hummingbird's diet (Levey & Martínez del Río 2001).

Gender differences are unlikely to be caused by metabolic differences, and physiological differences between genders have not been studied in relation to protein, although they have been examined in relation to sugar digestion (Markman et al. 2006). Both body size (Sweeting et al. 2007, Jennings et al. 2008) and metabolism (MacAvoy et al. 2006, Jennings et al. 2008, Martínez del Río et al. 2009) can affect nitrogen isotopes. However, in captive studies of other birds, there has been no indication that ^{15}N assimilation differed between genders (Pearson et al. 2003). Some captive studies can be difficult to extrapolate to natural environments, but metabolic pathways are unlikely to be affected by captivity.

Because this is a relatively new field, there is also a possibility of other unexplained effects. A more precise baseline of $\delta^{15}N$ for the ecosystem would strengthen the conclusions presented here. For example, if the spiders I sampled were actually representative of the higher trophic levels of prey items, we would have expected feather values to have $\delta^{15}N$ values 2.4 ‰ higher than the arachnids, rather than similar values. Clearly, the full explanation of sources of assimilated protein sources for these hummingbird communities is more complex than this research could detect. In fact, it is difficult to account for all of the sources of variation even in much better-studied systems (Daugherty & Briggs 2007). Further research should pay careful attention to achieving a representative sample of all potential protein sources.

In addition to the hypotheses this study was designed to address, I found an unanticipated direct relationship between elevations and feather $\delta^{15}N$ for *A. melanogenys* (the species with the widest altitudinal range). Such an effect has not been reported (much less explained) by any study using N-15. The present study was not designed to test for an effect of elevation. Nevertheless, this relationship should be examined in future studies that include samples form a broad elevation range to determine whether it might represent a general phenomenon. Because I did not determine the sex of the *A. melanogenys* I captured, it is possible that my small sample of this species at the highest elevation consisted of only females. If female *A. melanogenys*, like the females of the other species, have higher $\delta^{15}N$ values, then the relationship to elevation might reflect different gender ratios at different sites, resulting from partial migrations. Alternatively the selection of arthropods possibly might vary with site, and affect the $\delta^{15}N$ of assimilated protein. It is therefore possible that this relationship might be an artifact of sampling, but it might also have a biogeochemical explanation. In any case, it is important to report and understand, since such a relationship could confound results of other studies. Very few studies have examined the effect of elevation on δ15N, and none anywhere near this system, but changes in soil $\delta^{15}N$, total N, and gravimetric water content have been found to affect leaf $\delta^{15}N$ elsewhere (Bai et al. 2009).

Stable isotope analysis offers novel insights into the diets of nectarivorous birds, and overcomes some of the challenges that have plagued behavioral studies, like the difficulty in getting gender-specific and arthropod foraging data. In this paper, I have shown strong differences between the diets of male and female hummingbirds, which I suggest are the product of both physiological constraints of sexual selection, and the protein requirements of breeding. These differences have been suggested previously, but have not been convincingly confirmed until this paper.

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