The integration of energy and nitrogen balance in the hummingbird *Sephanoides sephaniodes*

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Summary

Floral nectars are rich in energy but contain only traces of amino acids, and it has been hypothesized that arthropods may be an important source of energy and amino acids for hummingbirds. We studied the nitrogen requirements of hummingbirds as well as how they use small arthropods to satisfy their nitrogen and energy requirements and how organ mass varies with nitrogen intake. Non-reproductive green-backed firecrowns *Sephanoides sephaniodes* were maintained for 10 days on diets containing 0%, 0.96%, 1.82%, 4.11% and 11.1% nitrogen (dry-matter basis). A second group of individuals were fed with varying amounts of nitrogen-free nectar supplemented with fruit flies. Finally, non-reproductive hummingbirds were captured as a control group for analysis of organ mass and size as well as fat content.

The maintenance nitrogen requirement of green-backed firecrowns determined by regression was 1.42 mg N day^{-1}, yet they required nearly 10 mg N day^{-1} to maintain body mass. When arthropods were available, we observed that hummingbirds required approximately 150 fruit flies to maintain body mass, which corresponds to a 5% nitrogen diet. Interestingly, when nectar was restricted (to 4 ml day^{-1}), or was absent, arthropods alone were not able to satisfy the body mass balance requirements of hummingbirds, suggesting that arthropods are not adequate as an energy source. In the group offered an 11.1% nitrogen diet, the size and surface of the small intestine, and liver and kidney mass increased in comparison with the control group (non-reproductive field hummingbirds) or the nitrogen-free group, suggesting a nitrogen overload. Our results are in agreement with other studies showing low nitrogen requirements by nectarivores. An important point to stress is that nitrogen digestibility declined in the 11.1% nitrogen diet, which strongly supports our nitrogen absorption saturation hypothesis.

Key words: nitrogen balance, energy balance, food quality, arthropod consumption, hummingbird, green-backed firecrown, *Sephanoides sephaniodes*, Chile.

Introduction

Floral nectars exploited by hummingbirds are rich in water and carbohydrates, but contain only traces of amino acids, minerals and vitamins (Baker and Baker, 1973; Gottsberger et al., 1984). Theoretically, the nitrogen found in most floral nectars is insufficient to meet the requirements of nectarivorous vertebrates (Baker, 1977; Baker and Baker, 1973; Law, 1992; Martínez del Río, 1994). Hummingbirds may therefore require alternative protein sources. Most, if not all, hummingbird species examined to date occasionally feed on arthropods (Bent, 1940; Remsen et al., 1986; Stiles, 1995). Many observations suggest that hummingbirds only invest between 2% and 15% of their daily time budgets in consuming insects (Gass and Montgomerie, 1981; Pyke, 1980). However, other studies report hummingbirds spending as much as 70% of their time foraging for insects (Wolf, 1970) and that they may even feed themselves exclusively on this resource (Hainsworth, 1977; Des Granges, 1978; Montgomerie and Redsell, 1980; but for criticisms, see Stiles, 1995). Indeed, as

pointed out by Stiles (1995), these contradictory results suggest that the real importance of arthropods for the foraging and nutritional ecology of hummingbirds is still unknown. Arthropods have high energy content (Bell, 1990), and are well assimilated by insectivores (Karasov, 1990). It has been suggested that, in addition to providing nitrogen and other essential nutrients, arthropods may be an important source of energy for hummingbirds (Hainsworth, 1977; Gass and Montgomerie, 1981). To our knowledge, this hypothesis has not been tested by a rigorous experimental approach.

Robbins (1993) calculated the minimum maintenance nitrogen requirements (MNR) of domestic and wild birds (430 mg N kg$^{0.75}$ day^{-1}). Recently, MNR values for three small (Brice and Grau, 1991; McWhorter, 1997; McWhorter et al., in press) and two medium sized (McWhorter et al., in press) species of hummingbirds have been reported. Interestingly, hummingbirds appear to exhibit lower MNR values than predicted by Robbins (1993). These low requirements appear

to be correlated with food habits, as similar low values have been observed in nectarivorous honeyeaters and sunbirds (Paton, 1982; Bradshaw and Bradshaw, 2001; Roxburgh and Pinshow, 2000) as well as in frugivorous birds and small mammals (Howell, 1974; Smith and Green, 1987; Izahaki, 1992; van Tets and Nicolson, 2000; Roxburgh and Pinshow, 2000).

Several studies have demonstrated that hummingbirds regulate their energy balance on a daily scale and that any energy imbalance immediately affects their behavior and body mass maintenance (Hainsworth, 1978; Wolf and Hainsworth, 1980; Calder et al., 1990; Martínez del Río and Karasov, 1990; McWhorter and López-Calleja, 2000; Fernández et al., 2002; López-Calleja and Bozinovic, 2003). Nevertheless, few studies have examined the protein balance of hummingbirds over a longer period than a daily time scale. Brice and Grau (1991) observed that Costa's hummingbirds had a reduced body mass after 5 days without feeding on a protein source, suggesting that a lack of protein affects hummingbirds more slowly than does a lack of energy.

In this study, we tested the effects of nitrogen intake on nitrogen balance, and the relationship between nitrogen and energy balance in a medium sized (ca. 6 g) hummingbird, the green-backed firecrown *Sephanoides sephaniodes*. The green-backed firecrown is a migratory hummingbird that visits the semi-arid Mediterranean-like environments of central Chile during the austral fall and winter (Goodall et al., 1956). The sugar concentration in floral nectar consumed by this species ranges from 0.3 to 1.2 mol l^{-1} (Belmonte, 1988; Smith-Ramirez, 1993), which is within the range of previously reported sugar concentrations of nectars in typical hummingbird flowers (Baker, 1975; Hainsworth and Wolf, 1976; Pyke and Waser, 1981). Our study focused on four objectives: (1) to determine if the MNR of this species is similar to that of other hummingbirds; (2) to analyze the effect of different dietary nitrogen concentrations on nitrogen and energy balance; (3) to determine if a diet composed exclusively of arthropods allows hummingbirds to maintain energy balance; and finally (4) to document the effect of varying dietary nitrogen concentration on digestive anatomy and body composition.

Materials and methods

Animals and their maintenance

During autumn 2000, mature male green-backed firecrowns *Sephanoides sephaniodes* Molina, N=38, body mass M_b=6.22±0.47 g (mean ± S.D.) were captured with mist nets at El Pangue (70°50′W, 33°31′S), in central Chile. Hummingbirds were maintained at an ambient temperature (T_a) within their thermoneutral zone (28±2°C; López-Calleja and Bozinovic, 1995) in separate cages (0.60 m×0.60 m×0.60 m) in an experimental room with natural and artificial light (12 h:12 h, L:D). Water and artificial nectar (0.75 mol l^{-1} sucrose in distilled water, supplemented with minerals, vitamins and proteins (1.5 g/100 ml of solution, ProMod®; Abbott, Columbus, USA) were provided *ad libitum*.

All experiments were conducted during the non-reproductive period. Hummingbirds were acclimated to experimental cages for 2 days before experiments began, and body mass variations were recorded during this period. Only those birds that maintained or increased M_b were included in experiments.

Nitrogen requirements with artificial diet

To determine nitrogen requirements, hummingbirds were randomly assigned to five different groups, which were provided with the following dietary nitrogen concentrations: N-0 (N-0), 0.96% (N-1), 1.82% (N-2), 4.11% (N-5) and 11.1% (N-11). Diets were isocaloric by the interchange of protein and sucrose as required. The amounts of minerals and vitamins were similar in the diets (Table 1). During the acclimation and experimental periods, diets were offered *ad libitum* in 10 ml syringes, and were changed twice or three times a day to avoid protein precipitation. Water was offered *ad libitum* in 3 ml syringes and in plates that birds also used for bathing.

Food intake and body mass change

To determine food intake, birds were maintained for 11 days on each diet. Volumes of food and water intake (ml day^{-1}) were recorded 2–3 times a day. To correct for evaporative losses, control feeders not accessible by birds but containing the different diets and water were located just outside the cages and recorded at the same intervals. Body mass changes were recorded daily (before 07:00 and after 18:00 h) with an analytical balance (accuracy±0.01 g; Acculab V-200, Edgewood, USA). Both food consumption and body mass were recorded throughout the acclimation period.

Nitrogen and energy balance

Nitrogen (N) and energy balance were determined during the last 2 days of the acclimation period, and during the 4th and 5th days for the free access-diet group (see below). To determine nitrogen and energy balance, early in the morning, birds were moved to identical experimental cages, which were lined with plastic sheeting along the bottom and walls to collect excreta. During daylight periods, excreta were collected hourly and immediately frozen to minimize nitrogen loss (see below).

Table 1. *Dietary composition of experimental diets*

	Diets				
	N-0	N-1	N-2	N-5	N-11
Number of individuals					
Initial	12	10	6	6	6
Final	11	8	6	6	6
Composition of diet					
Sugar (g)	262.0	260.0	252.9	244.0	220.7
Nitrogen (g)	0	0.96	1.82	4.11	11.1
Vitamins (g)	0.071	0.071	0.071	0.071	0.071
Energy (kJ g^{-1})	16.49	16.56	16.63	16.74	17.15

The number of individuals in each dietary group and the chemical composition of the artificial diets are indicated.

Excreta from night time were collected early in the morning with a rubber spatula and a small amount of water. Food and water intake were also monitored. To determine nitrogen and energy contents, respectively, samples of food and all collected excreta were analyzed in duplicate using the micro Kjeldahl semi-automated method for nitrogen determination and a computerized calorimeter (Parr 1261; Moline, USA). Two replicates were determined to be ash free and reliable when the difference between values was less than 1%. Considering that nitrogen losses, especially gaseous ammonia, could be important during the collection and drying process of excreta, we followed the protocol developed by Roxburgh and Pinshow (2000), that is, fresh excreta samples that were immediately frozen were compared with samples that were frozen after 1 h of exposure to air on plastic sheets. These latter samples contained 89.9±1.8% (N=5) of the nitrogen found in the immediately frozen samples and nitrogen excretion. Data were corrected for nitrogen losses using this value.

Nitrogen retention (i.e. assimilated nitrogen) was calculated as N_i – total N_o (mg N day^{-1}), where N_i = N intake and N_o = N output. Assimilated N was plotted against dietary N intake, and the regression for N balance was calculated. Food intake and excreta produced during 24 h were measured to estimate apparent assimilated mass coefficient, $AMC^* = (Q_i – Q_e)/Q_i$, where Q_i and Q_e are dry food intake and excreta production rates in g day^{-1}, respectively (Karasov, 1990). AMC^* is apparent because birds mix urinary and fecal products in the hindgut and eliminate them together *via* the cloaca (Robbins, 1993). We also calculated digestible energy intake (DE_i) and digestible nitrogen intake (DN_i). DE_i was calculated as $Q_i \times GE_i \times AMC^*$ (kJ day^{-1}), where GE_i is the gross energy content of the diet, and DN_i as $Q_i \times GN_i \times AMC^*$ (mg day^{-1}), where GN_i is the gross nitrogen content of diets. Thus, AMC^* was calculated for both energy and nitrogen.

Organ masses

To evaluate the effects of protein intake on digestive organ morphology, the mass and fat content of internal organs were determined after the nitrogen balance trials were completed. Morphometric variables of birds from groups N-0, N-2 and N-11 were compared with birds captured in the field during the same period (winter). Animals were killed by cervical dislocation. We measured organ size and mass according to Sabat and Bozinovic (2000) and Konarzewski and Diamond (1994, 1995). Wet length and nominal area of the total intestine from the end of gizzard to the cloaca were measured to the nearest mm. The nominal surface area of the small intestine was determined by multiplying mean luminal circumference (measured at three equidistant points) by length. Mesenteries and fat were removed prior to measurement to ensure maximum extension when suspended from one end. Dry mass (after removal of adherent fat) of carcass, heart, kidneys, liver, intestine and gizzard were also determined after drying to constant mass in an oven at 65°C. Adherent and carcass fat of hummingbirds were measured according to Konarzewski and Diamond (1994) except that rather than samples we used the entire bird. Ash of carcass was determined by burning samples in a muffle oven for 3 h at 500°C. Lean fresh muscular mass LFMM was calculated as carcass body mass minus the fat and ash contents.

Nitrogen and body mass balance on simulated natural diets

To determine the effects of variation in nectar quantity on the number of arthropods consumed we used fruit flies *Drosophila melanogaster*, which are similar in size to insects consumed by hummingbirds in the field (M. V. López-Calleja, unpublished data). We designed a sequential protocol with three different experimental situations. First (Nect+FF group), over a period of 5 days we determined the nectar/protein ratio used by six green-backed firecrowns given fruit flies and N-0 nectar *ad libitum*. During the pre-acclimation period, the photoperiod and thermal conditions were similar to the artificial diet experiments described above. On the last day we collected excreta using the same protocol as that in the artificial diet experiment. In the second sequence (Nect$_4$+FF group), we attempted to determine if M_b balance would be maintained with a restricted nectar diet. We used the same birds acclimated to eat fruit flies and nectar, and during one day we offered a limited amount of nectar (3 ml during early morning and 1 ml in the afternoon). In the final sequence (FF alone group), and after birds had recovered M_b (2 days later), we explored whether hummingbirds were able to maintain M_b eating only fruit flies. Throughout all of these sequences we recorded variation in M_b, the number of fruit flies and volumes of nectar and water consumed, and finally the presence of torpor during night. To measure fly consumption, we collected 500 live fruit flies per hummingbird and released them into the experimental cages before the light turned on in the morning. When the light turned off, we moved each bird to its nocturnal cage and counted the remaining fruit flies.

Statistics

Statistical analyses were performed using the STATISTICA (1997) release 5 for Windows 95 (third edition; StatSoft, Inc., Oklahoma, USA). We used two-way as well as repeated-measures analysis of covariance (ANCOVA) with body mass (M_b) as a covariate. To satisfy the assumptions of these parametric statistical methods, we transformed digestibility and fat data to arcsine square root values (Zar, 1997). During the acclimation period, paired sample Student's *t*-tests for related measurements with Bonferroni corrections were used to test for daily changes in M_b, as well as in food and energy intake. All results are reported as mean ± 1 standard error (S.E.M.).

Results

Body mass and food intake on artificial diets

At the beginning of this experiment, M_b did not vary among experimental groups (6.13±0.07 g; ANOVA, $F_{4,30}$=0.89; P=0.48). Protein challenge, however, significantly affected the dynamics of M_b (Fig. 1). Body mass of hummingbirds
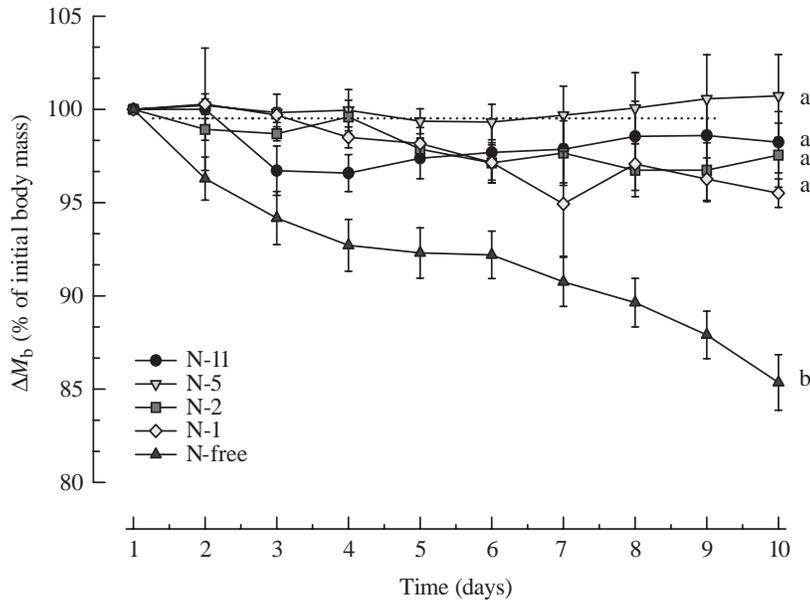
Fig. 1. Body mass M_b change of green-backed firecrowns *Sephanoides sephaniodes*, fed diets with different nitrogen contents (see text) over a 10 day acclimation period. M_b of N-0 group changed significantly after treatment. ΔM_b was significantly different between groups at the end of the acclimation period. Different letters indicate significant differences between groups after an a *post-hoc* Tukey test. The dotted line represents no change in body mass.

maintained on the N-0 diet began to decrease during the second day of acclimation (paired samples, Student *t*-test, t_{10}=3.424, P=0.007). The slopes for the birds on the N-0 and N-1 diets were negative and significant (ANOVA N-0: $F_{1,108}$=92.25,

P<0.0001 and ANOVA N-1: $F_{1,78}$=19.66, P<0.001), i.e. M_b decreased in birds on both diets during acclimation, but clearly the N-0 group lost mass faster than the N-1 group. The slopes for the other groups were not significantly different from zero (ANOVA, P>0.05). Consequently, significant differences in M_b were observed between groups at the end of the acclimation period (Table 2). Animals from N-2, N-5 and N-11 maintained M_b (paired samples, Student *t*-test, N-2: t_5=0.92, P=0.40; N-5: t_5=–0.58, P=0.58; N-11: t_5=–0.09, P=0.93), while in the other two groups a significant decrease in M_b was recorded (paired samples, Student *t*-test, N-0: t_{10}=5.14; P<0.001; N-1: t_7=5.65; P<0.01).

The volume of food (ml day^{-1}) and energy (kJ day^{-1}) intake were similar between birds on different diets at the beginning of the acclimation period (ANCOVA; $F_{4,30}$=0.63, P=0.64, and $F_{4,30}$=0.82, P=0.52, respectively), with a mean of 11.24±0.40 ml day^{-1} or 46.66±1.66 kJ day^{-1}. At the end of the experiment, food and energy intakes showed significant variation as a function of dietary nitrogen content (Table 2). Hummingbirds in groups N-11 and N-5 consumed more food and gross energy in comparison to birds in the N-1 and N-0 groups. Energy digestibility was different between diets, with the N-11 diet exhibiting the lowest digestibility (Table 2). Digestible energy intake (DE$_i$) followed the same general pattern as gross energy intake. Only the extreme diets were significantly

Table·2. *Body mass and food intake of* Sephanoides sephaniodes *during the last day of acclimation on artificial diets of different nitrogen concentration*

	Diet (% N)					ANCOVA*	
	N-0 (0%)	N-1 (1.2%)	N-2 (2.3%)	N-5 (4.7%)	N-11 (11.2%)	$F_{4,30}$	P
Number of birds:	10	8	6	6	8		
Body mass M_b (g)							
Last day	5.26±0.16[a]	5.54±0.06[b]	6.13±0.02[b]	6.30±0.12[b]	6.24±0.21[b]	24.95	<0.0001
Daily increase	0.77±0.10[a,b]	1.11±0.11[a,b]	0.65±0.11[a]	0.56±0.08[a]	1.02±0.10[b]	4.52	0.006
Final – initial	–0.91±0.09[a]	–0.36±0.05[b]	–0.11±0.15[b]	0.07±0.14[c]	0.01±0.08[b,c]	7.38	<0.001
Food intake (ml·day^{-1})	7.65±0.16[a]	7.89±0.74[a]	10.05±0.09[a,b]	10.33±0.11[a,b]	12.3±0.22[b]	5.45	0.001
Energy							
Intake (kJ·day^{-1})	31.97±2.86[a]	33.27±3.32[a,b]	42.72±3.47[b,c]	44.52±3.31[c]	53.01±2.93[c]	6.39	<0.001
Digestibility (%)	0.99±0.001[a]	0.98±0.001[b]	0.98±0.002[b]	0.97±0.001[b]	0.93±0.002[c]	65.2	<0.001
Assimilated (kJ·day^{-1})	31.33±2.86[a]	32.91±4.42[a,b]	41.87±3.60[a,b]	43.27±3.26[a,b]	48.55±3.27[c]	5.19	0.002
Nitrogen							
Intake (mg·day^{-1})	0[a]	5.16±0.42[a,b]	9.26±0.65[b]	17.56±1.41[c]	50.06±4.52[d]	87.49	<0.001
Digestibility (%)	–	0.59±0.04[a,b]	0.68±0.07[a]	0.55±0.03[a,b]	0.48±0.03[b]	5.77	0.005
Assimilated (mg·day^{-1})	–1.33±0.16[a]	2.89±0.40[b]	6.80±0.66[b,c]	10.96±1.23[c]	22.92±1.61[d]	71.02	<0.001

Numbers in parentheses in the first row indicate final number of individuals assigned to each treatment.
Values are means ± 1 s.e.m. and different letters show significant differences (Tukey test).
*Significant differences between diets were tested using one-way ANCOVA with final body mass as covariate.

different (N-0 *versus* N-11, Table 2). Interestingly, N-0 individuals reduced their food and energy intakes with respect to the observed initial values ($Z=2.57$, $P=0.009$ for both variables) and those of the N-11 group, despite the fact that they presented the highest energy digestibility (Table 2). Nitrogen intake and assimilation were significantly different between diets at the end of the acclimation period (Table 2), but nitrogen digestibility decreased in the most concentrated diet (Table 2).

The observed increase in M_b during daylight hours changed during the experimental period according to diet. Body mass increased on all diets during the first day (ANCOVA; $F_{4,30}=1.18$; $P=0.31$), mean ± S.E.M. = $0.84±0.06$ g day^{-1}. At the end of the acclimation period, however, the diurnal increase in M_b was significantly different between groups (ANCOVA; $F_{4,30}=4.52$; $P=0.006$). Body mass of birds in the N-11, N-1 and N-0 treatment groups increased at a higher rate than those in groups N-5 and N-2 (Table 2).

Body mass and food intake variation: diets with natural nitrogen sources

All groups fed fruit flies showed similar M_b at the beginning (Table 3), but this pattern changed during the 5 days of experimentation. Hummingbirds in the group fed N-0 nectar plus fruit flies *ad libitum* (Nect+FF) maintained constant M_b (paired sample Student's *t*-test, $t_5=0.77$, $P=0.49$) and nectar intake (paired sample Student's *t*-test, $t_5=-1.78$, $P=0.27$) during the experiment. Fruit fly intake increased during the second day (paired sample Student's *t*-test, $t_5=-9.06$, $P=0.001$), but declined and stabilized from the third until the fifth day (paired sample Student's *t*-test, $t_5=-2.86$, $P>0.01$). At

the end of acclimation, nectar intake was $10.1±0.3$ ml day^{-1}, and fruit fly consumption was $148±73$ flies day^{-1}, representing an energy intake of 45.45 kJ day^{-1}. Fruit flies represented approximately 9% of the total energy consumed.

Hummingbirds in the Nect$_4$+FF group decreased in M_b during daylight periods, however daily (24 h) changes in M_b were not significantly different from the Nect+FF group (Table 3). All hummingbirds assigned to the Nect$_4$+FF group entered into torpor at night, and consumed more fruit flies in comparison to the Nect+FF group, but consumed less energy (Table 3).

Individuals maintained only with fruit flies (FF alone), actively caught and ate insects during the first hours of the experiment. After 4 h, birds changed their activity pattern, remaining perched with feathers ptiloerected, a behavior presumably to reduce thermoregulatory costs. Considering only the time while the birds were active, the number of fruit flies eaten per hour was $14.8±3.5$, a value similar to that observed in the other two natural diets (Nect+FF and Nect$_4$+FF, see Table 3). Nevertheless, hourly energy intake was significantly lower for FF group; in fact birds consumed only 12% of the energy consumed by individuals assigned to the Nect+FF treatment (Table 3).

Fig. 2 shows M_b change and nitrogen assimilation in both experimental and natural dietary treatments. Body mass changes in the Nect+FF group were lower in comparison to the N-0 experimental group (ANCOVA $F_{5,34}=8.24$, $P<0.0001$, Fig. 2A). Nitrogen assimilation for the Nect+FF group was $4.55±0.24$ mg N day^{-1} (or $148±7$ fresh fruit flies), a value similar to that observed in individuals assigned to the N-2 and N-1 dietary groups (ANCOVA $F_{5,34}=65.10$, $P<<0.0001$; see

Table 3. *Body mass, energy and fruit fly intake of* Sephanoides sephaniodes *acclimated to offered natural diets differing in nitrogen quantity*

	Groups				
	Nect+FF	Nect$_4$+FF	FF alone*	*F*	*P*-value
Number of birds:	6	6	5		
Body mass (g)					
Initial	5.69±0.08	5.56±0.09	5.40±0.09	2.16	0.16
After 12 h – initial	0.51±0.02	–0.12±0.01	–	33.45	0.0004
After 24 h – initial	–0.06±0.05	–0.21±0.04	–	0.02	0.18
Nectar intake (ml day^{-1})	10.06±0.30	4.00±0	0[†]	3.48	0.03
Fruit fly intake					
Number day^{-1}	147.6±7.20[a]	171.3±6.85[b]	81.4±12.9[†]	5.35	0.05
Number h^{-1}	12.8±0.57	14.9±0.61	16.7±2.50	0.67	0.53
Energy intake					
kJ day^{-1}	45.45±1.15	21.19±0.19	2.41±0.39	686.15	<0.0001
kJ h^{-1}	3.95±0.22[a]	1.84±0.04[b]	0.46±0.16[c]	704.64	<0.0001

Values are mean ± 1 S.E.M.

F and *P* values are from a one-way ANCOVA, using body mass as a covariate.

Diets: Nect+FF, nectar and fruit flies *ad libitum*; Nect$_4$+FF, 4 ml of nectar and fruit flies *ad libitum*; FF alone, only fruit flies.

*Each bird was kept on this diet as long as it could maintain its normal pattern of activities (4–6 h approx.).

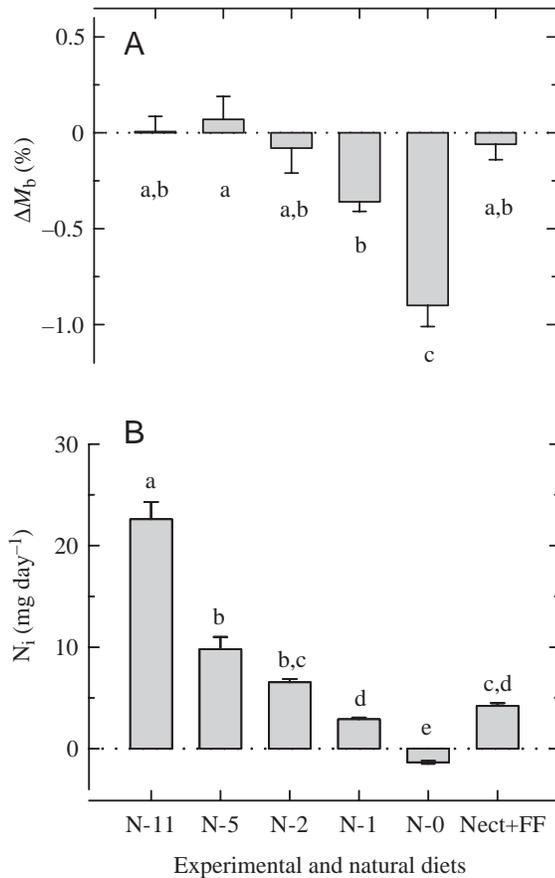[†]For information only; this value was not used in the analyses.

Fig. 2. (A) Body mass M_b change and (B) nitrogen assimilation N_i by *Sephanoides sephaniodes* on experimental diets with different concentrations of nitrogen and on a natural diet with *ad libitum* N-0 nectar and fruit flies (Nect+FF). Values are means ± S.E.M. Different letters indicate significant differences between groups after a *post-hoc* Tukey test.
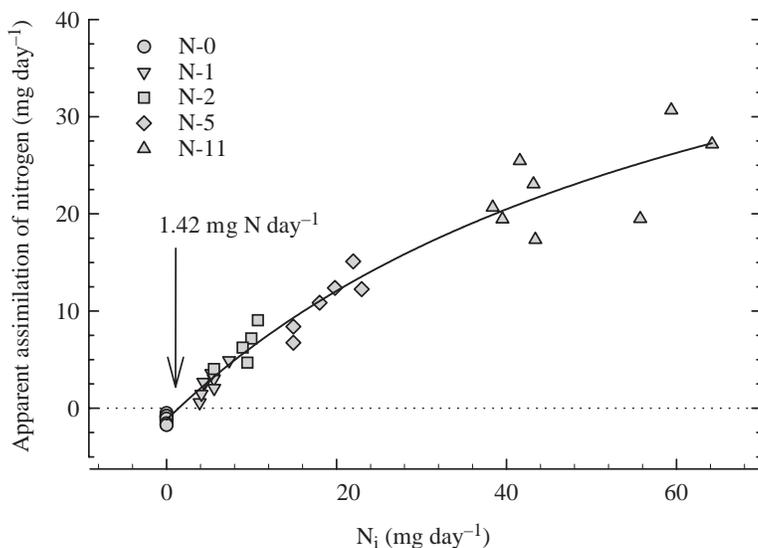
Fig. 2B for *post hoc* comparisons). Nitrogen digestibility on the natural diets was 78.8±6.4%, higher than that observed in all the experimental groups (see Table 2). The energy intake of the Nect+FF group was 45.45±1.15 kJ day^{-1} (Table 3), similar to that observed on the N-2, N-5 and N-11 experimental diets (nectar intake: ANCOVA, $F_{6,34}$=5.28, P=0.001; energy intake: ANCOVA, $F_{6,34}$=2.60, P=0.04; Table 3).

Nitrogen balance

The amount of N required for hummingbirds to maintain a positive N balance was determined by a regression between the apparent N retention on N intake for all 31 birds from all artificial dietary treatments (Fig. 3). Considering that nitrogen assimilation was significantly different between diets, and that birds presented an apparent saturation of intake capacity on the highest N diet (N-11, Table 2), we selected the best curve fitting all data. Birds were in positive balance (MNR) at an average of 1.42 mg N day^{-1} [y=−1.19+(59.16x/69.22+x), r^2=0.96, P=0.001] or 67.92 mg N kg$^{-0.75}$ day^{-1}, corresponding to the experimental nitrogen content of the N-1 diet (1.2% of dietary nitrogen).

Total endogenous nitrogen losses (TENL), measured directly (i.e. on a N-0 diet) were −1.33±0.16 mg N day^{-1} or 67.86±8.33 mg N kg$^{-0.75}$ day^{-1} (Table 2). In most studies, endogenous nitrogen losses are not measured directly because few animals will consume nitrogen-free diets. The equation of regression between the apparent N retention on N intake from all artificial dietary treatments predicted a TENL of 1.19±0.51 mg N day^{-1} or 55.20±23.66 mg N kg$^{-0.75}$ day^{-1}. This value is in agreement with the value measured directly. Nevertheless, when changes in body mass during the entire experiments were regressed against daily nitrogen intake (*sensu* Murphy, 1993), we estimated that 5.83±1.4 mg N day^{-1} was necessary to maintain body mass for *Sephanoides sephaniodes* (r^2=0.93, P=0.001). This value was higher than the values obtained by MNR or TENL, but is consistent with the observation that animals in the N-2 group maintained body mass.

Energy balance

Digestible energy intake (DE$_i$) was significantly different between artificial and natural diets (Tables 2, 3). Animals assigned to N-0 and N-1 decreased DE$_i$ and M_b. To confirm that the volume of N affected DE$_i$ and M_b, we conducted a linear regression of DE$_i$ against overall change in M_b (Fig. 4), thus estimating that *S. sephaniodes* needs 43 kJ day^{-1} to maintain M_b. This value is not

Fig. 3. Apparent nitrogen retention of green-backed firecrowns *Sephanoides sephaniodes* fed on different experimental diets containing different nitrogen concentrations (see text). Regression equation is y=−1.19+59.16x/(69.22+x), r^2=0.96, P<0.001. Nitrogen balance (or minimum maintenance nitrogen requirement, MNR, when y=0), occurs at an intake of 1.42 mg N d^{-1}.
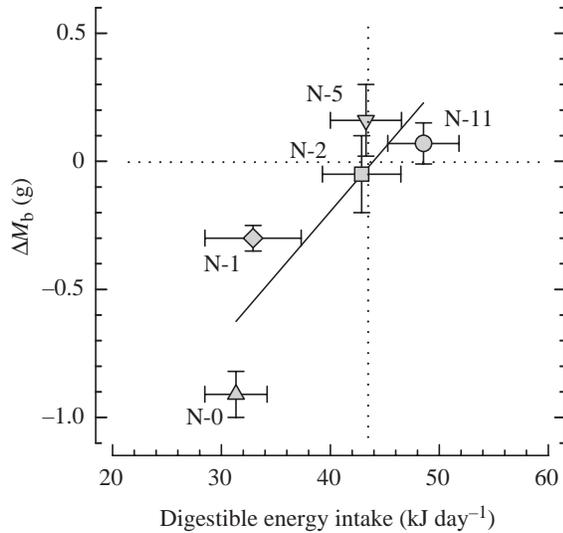
Fig. 4. Linear regression of digestible energy intake (DE_i) against overall change in the M_b of *Sephanoides sephaniodes* during 10-day feeding trials on diets of different nitrogen concentrations (see text). Regression equation is $y=0.05x–2.15$, $r^2=0.78$, $P<0.05$. The DE_i (44.0 ± 1.1 kJ day^{-1}) and ΔM_b ($–0.06\pm1.1$ g), observed in birds fed the fruit flies and nectar diet *ad libitum* is indicated by the dotted lines.

statistically different to the one in the experiments with fruit flies (Nect+FF) and artificial diets N-2 and N-5.

Morphological effects of nitrogen intake

Body and organ masses are presented in Table 4. The N-0 group clearly exhibited lower body, carcass and free-fat

carcass masses than birds in the N-11 and control groups. Fat as a percentage of total body mass was higher in birds in N-0 than N-11 and control groups. Feather mass was similar among all groups. The masses and sizes of several organs changed significantly between the different artificial dietary treatments as well as in comparison to the control group (Table 4). Kidney, gizzard and small intestine (mass and length) decreased in individuals assigned to the N-0 diet in comparison to controls (Table 4). An increase in liver mass was observed only in hummingbirds from the N-11 group. Kidneys of N-0 and N-2 individuals were lower in mass in comparison to controls (Table 4).

Discussion

A major goal in physiological ecology is to understand and explain the extrinsic and intrinsic factors that may impose limits to an animal's mass and energy budget and to quantify how individuals acquire and utilize energy and nutrients. To this end, we studied the energy and nitrogen requirements of hummingbirds, together with the implications for nitrogen and energy economy, body mass balance, fat content and organ masses. We recorded low nitrogen requirements that may be an adaptation to low fat, fiber-free diets; however, hummingbirds cannot maintain body mass when feeding on nitrogen-free diets.

Nitrogen requirements

The minimal nitrogen requirement of *Sephanoides sephaniodes* recorded in this study was 1.42 mg N day^{-1} or 65.87 mg N kg$^{-0.75}$ day^{-1}. These values are similar to the

Table 4. *Body mass and organ size of* Sephanoides sephaniodes *acclimated to different nitrogen intakes and in field conditions*

	Diet					
	N-0	N-2	N-11	Control field	F	P-value
Number of birds:	6	5	7	6		
Body mass (g)	5.61±0.22[a]	6.09±0.17[a,b]	6.47±0.25[b]	6.53±0.22[b]	3.84	0.02
Carcass	1.55±0.10[a]	1.99±0.17[a,b]	1.98±0.18[a,b]	2.06±0.05[b]	3.31	0.04
Feathers	0.51±0.02	0.48±0.02	0.53±0.02	0.49±0.02	1.16	0.35
Fat	0.63±0.10	0.63±0.05	0.50±0.07	0.58±0.03	0.43	0.73
Carcass without fat	0.92±0.07[a]	1.36±0.10[a,b]	1.42±0.12[b]	1.49±0.08[b]	11.23	<0.001
Organs (mg dry mass)						
Heart	32.07±2.22	35.00±0.71	36.13±1.46	30.90±1.00	2.77	0.07
Lung	19.24±3.11	15.77±0.79	19.45±1.77	19.22±1.33	0.46	0.71
Kidney	10.62±1.36[a]	14.15±1.01[a,b]	17.60±0.78[b,c]	19.90±2.03[c]	5.50	0.007
Liver	43.87±7.64[a]	47.87±4.24[a]	83.32±13.97[b]	54.47±3.73[a]	6.92	0.003
Gizzard	2.67±0.09[a]	3.30±0.27[a,b]	3.77±0.15[b]	3.92±0.15[b]	7.62	0.002
Small intestine						
Mass (mg)	32.89±4.30[a]	33.10±4.03[a]	52.52±2.01[b]	49.2±2.40[b]	5.26	0.009
Length (mm)	58.33±2.70[a]	65.00±0.41[a,b]	75.33±0.98[c]	71.67±2.17[b,c]	12.54	0.0001
Nominal area (mm^2)	275.26±9.95[a]	276.17±9.78[a]	370.84±19.59[b]	328.71±16.39[a,b]		0.003

Values are mean ± 1 s.e.m. Different letters show significant differences (Tukey test).
Body and organ mass were recorded after the 12th day of acclimation.
F and P values are from a one-way ANCOVA, using body mass as covariate.
Diets: N-0, 0% nitrogen; N-2, 2.3% nitrogen; N-11: 11.2% nitrogen.

measured TENL (1.32 mg N kg$^{-0.75}$ day^{-1} or 67.86 mg N kg$^{-0.75}$ day^{-1}), which is the combination of endogenous urinary and metabolic fecal nitrogen loss. These values represent the minimum amount of nitrogen that an animal would need to replace in order to maintain a positive nitrogen balance.

Both MNR and TENL values are approximately 15% of the values predicted by Robbins (1993), 430 mg N kg$^{0.75}$ day^{-1} or 9.27 mg N day^{-1} for a bird of this body size. Also, MNR and TENL were significantly lower than the N requirements for M_b maintenance estimated according to Murphy (1993). *Sephanoides sephaniodes* maintained M_b when fed either Nect+FF (with fruit flies to fulfil their nitrogen requirements), or the artificial N-2 diet. Interestingly, both diets exceed their MNR requirements. This situation is similar to that reported in other studies. Brice and Grau (1991), Murphy (1993) and Roxburgh and Pinshow (2000) reported that small nectarivorous birds were only able to maintain M_b on diets that substantially exceed their MNR. Apparently a fair proportion of the amino acid content of the food consumed does not exactly match the animal's amino acid requirements (Robbins, 1993). Birds are unable to synthesize 10 of the 20 or so obligatory amino acids (Murphy, 1996). Thus, diets with low nitrogen concentration may not match the specific nutritional requirements of the species. As an example of this mismatch, Bradshaw and Bradshaw (2001) proposed that a dietary amino acid deficiency may explain the reduced rate of reproduction observed in the honey possum *Tarsipes rostratus*. Alternatively, the difference between MNR and M_b maintenance requirements could be explained by sloughed skin or feathers or ammonia lost over respiratory surfaces as suggested by Brice and Grau (1991) and Roxburgh and Pinshow (2000).

TENL and MNR values in *S. sephaniodes* are very similar to those determined in other species of nectarivores (see Roxburgh and Pinshow, 2000; McWhorter et al., in press). Robbins (1993) hypothesized that animals feeding on liquid diets, which are also low in fiber and lipids (such as floral nectars), probably have low nitrogen losses due to lower secretion of protein digesting enzymes and bile acids, reduced sloughing of intestinal epithelial cells and smaller populations of gut microorganisms. Post-renal nitrogen recycling may explain the low MNR if hummingbirds are reducing losses of nitrogen in urate excretion and by recycling the protein associated with urate. Reduced urate in excreta may be the result of the breakdown of urates by uricotelic bacteria (McNabb et al., 1973; Dawson et al., 1991; Janes and Braun, 1997). Indeed, Roxburgh and Pinshow (2002) described a decrease in urate concentration and proportionally more ammonium in the excreta in the Palestine sunbird *Nectarinia osea* feeding on low nitrogen diets. Other workers, by contrast, have related the change in the ammonia:urate ratio to energetic constraints such as low temperatures rather than low dietary nitrogen concentrations (e.g. *Calypte anna*, Preest and Beuchat, 1997; *Pycnonotus xanthopygos*, van Tets et al., 2000). This explanation is based on a potential reduction in the metabolic cost of nitrogen excretion by excreting primarily ammonia.

The apparent nitrogen retention observed in *S. sephaniodes* feeding on the more N-concentrated diets suggests a saturation in nitrogen absorption capacity. The study of McWhorter et al. (in press) documents a nitrogen retention plateau in small hummingbirds feeding on high N diets. It is unclear if this is a byproduct of experiments where captive hummingbirds were fed artificial diets, because field data show that arthropods may be intensively consumed during short foraging bouts (Wagner, 1946; Hainsworth, 1977; Paton, 1982; Gass and Montgomerie, 1981). In the nectar of our experimental diets, nitrogen and carbohydrates are combined, unlike the diet in the field. If a very high nitrogen content were to increase gut transit time, then the daily volume of nectar that a hummingbird could eat would be reduced, with potentially negative effects on the daily energy balance. This explanation is in agreement with our experimental observations. Indeed, hummingbirds preferred liquid diets with lower nitrogen concentrations over more concentrated diets (M. V. López-Calleja, unpublished data). The adaptation to liquid diets, low in fiber and lipids, probably evolved together with a rapid gut transit time and high digestive efficiency that are typical of hummingbirds (Karasov, 1990; López-Calleja et al., 1997; McWhorter and López-Calleja, 2000). In general, the flow of the nectar in feeding birds is directly from proventriculus into duodenum, bypassing the gizzard altogether, whereas arthropods are diverted to the gizzard for mechanical maceration and peptic digestion (Klasing, 1998). Moreover, green-backed firecrowns have a large thin-walled crop, which always contained more arthropods than in other sections of the digestive tract (M. V. López-Calleja, unpublished data). We suggest that in natural conditions hummingbirds can transport arthropods gradually into the stomach, thereby not affecting carbohydrate absorption during the day.

Morphological effects

Body mass was lower in individuals from the N-0 group compared to individuals in control and N-11 groups (see Table 4), which may be explained by a significant decrease in muscular mass (fat-free carcass). Fat mass remained unchanged among groups, indicating that the decrease in M_b and food consumption at the end of our experiments may be explained by a nitrogen deficiency and not by an energy constraint.

Hummingbirds acclimated to N-0 diets or those with little nitrogen (N-0 and N-2) had shorter and lighter small intestines than birds in N-11 and control groups (see Table 3). Information about the effects of low dietary protein levels on the morphology of digestive organs in birds is scarce (Karasov, 1996). Nevertheless, according to Karasov (1990), Dykstra and Karasov (1992), Piersma et al. (1993) and McWilliams et al. (1999, 2001), the main adjustments in the digestive system that compensate for decreases in digestive efficiency are an increase in gut length, mass and/or volume (see also Bozinovic et al., 1990; Bozinovic, 1993a,b, 1995). Thus individuals

maintained on a high dietary protein load, such as those in the N-11 group, had longer and heavier intestines than the control group (field-caught hummingbirds). Hummingbirds were presumably consuming both nectar and arthropods before being captured, and arthropods contain indigestible matter, such as chitin, which may reduce digestive efficiency. It is possible that the dietary N levels experienced by the N-11 group reduced digestive efficiency to a greater extent than natural arthropod diets.

The increases in liver and kidney masses in hummingbirds exposed to high dietary nitrogen could be explained by an increase in the production and excretion of nitrogenous waste products resulting from a high protein load. Kidney hypertrophy has been documented in *S. sephaniodes* when exposed to chronic cold environments and diluted diets of high protein concentration (López-Calleja and Bozinovic, 2003), and in rodents confronted with high levels of protein in their diets (Klahr, 1989; Hammond and Janes, 1998).

Energy requirements: arthropods and nectar

Arthropods apparently cannot replace nectar as an energy source for hummingbirds. Our results demonstrated that when nectar was available in low abundance or was absent, *S. sephaniodes* consumed more fruit flies, but the total energy obtained was significantly lower than that when nectar was available (5–47% lower, see Table 3). Apparently, net energy gains when foraging for small arthropods are lower than when feeding on nectar diets. If arthropod consumption is limited by foraging constraints, then more larger prey items could possibly contribute enough energy. Dietary information about trophic preferences of *S. sephaniodes* during winter time indicates that the sizes of their prey are similar to the fruit flies offered in our experiment. Considering the energetic value of fruit flies, a green-backed firecrown would need to consume nearly 1700 flies in order to meet the energetic requirements observed in this study (more than 41 kJ day^{-1}). Nevertheless, in summer and at the southern limit of its distribution (near Puerto Williams, Chile, 54° 56′S, 67°37′W), *S. sephaniodes* does consume larger prey items than fruit flies (insects of 10–15 mm; R. Rossi, unpublished data). Everything else being equal, this size of prey would theoretically reduce the number of prey items required by hummingbirds in order to meet their energetic requirements. Several authors have documented that nesting hummingbirds consume more arthropods than they need to meet their nitrogen requirements, and suggested that these are used for long-term heat production during night time when food intake is suspended (Montgomerie and Redsell, 1980), probably avoiding torpor during incubation. Our results, however, indicated that *S. sephaniodes* obtains nearly 80% of its energy requirements from nectar carbohydrates during the non-reproductive period.

As Murphy (1996) indicated, the amino acids obtained from food are used mainly for replacement of basal losses or for synthesis of new tissues. Since the amino acids that are not used immediately are quickly catabolized (Heger and Frydrych, 1989), protein synthesis will require continuous availability of amino acids. Dietary deficiencies of amino acids have severe effects in birds, including reduced rates of protein synthesis and/or accelerated degradation, increased rates of oxidation, and reduced rates of ingestion of food deficient in amino acids (Murphy and Pearcy, 1993). In general, these limitations affect mass balance, and during chronic situations compromise survival (Murphy, 1996). In the N-0 treatment, *S. sephaniodes* dropped nearly 15% in M_b and reduced food intake at the end of the acclimation period. This pattern was not observed in previous work with hummingbirds (Brice, 1992; Brice and Grau, 1991) or other nectarivores (Roxburgh and Pinshow, 2000), where rates of nectar intake during a similar time span remained constant even when the protein concentration was reduced to zero. In both cases, birds lost 10–15% of M_b. Moreover, hummingbirds in short term N balance studies maintain or increase M_b (McWhorter et al., in press). Why *S. sephaniodes* decreased food consumption is not clear, but information from granivorous or carnivorous birds also indicated a decrease in food consumption for individuals on protein-diluted diets (Robbins, 1983; Murphy, 1996). We hypothesize that nitrogen balance in hummingbirds is regulated over a time scale of several days, as opposed to energy balance that is regulated on a day by day basis (Wolf and Hainsworth, 1980). Skeletal muscle is likely to be the first source of proteins used by birds exposed to a long-term absence of nitrogen (at least 10 days). A loss in M_b attributed to muscle mass would directly affect daily activity patterns, thereby compromising foraging ability and energy balance in hummingbirds.

Finally, animals in general, and hummingbirds in particular, can face unpredictability in food availability and quality at different times and periods. Indeed, the availability of both nectar and arthropods change seasonally as well as daily. We show that during the non reproductive – non growing period, *S. sephaniodes* require nearly 150 fruit flies per day to satisfy their full nitrogen balance and to maintain body mass; however that amount of flies represents a negligible energy supply.

An important point to stress is that nitrogen digestibility declined in the N-11 diet, which strongly supports our nitrogen absorption saturation hypothesis. Clearly, more laboratory and field oriented studies are necessary to understand how and when arthropods are relevant in the physiological and behavioral ecology of hummingbirds.

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