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ABSTRACT

Nectar-feeding birds have remarkably low nitrogen requirements. These may be due either to adaptation to a low-protein diet or simply to feeding on a fluid diet that minimizes metabolic fecal nitrogen losses. We measured minimal nitrogen requirements (MNR) and total endogenous nitrogen loss (TENL) in the omnivorous European starling *Sturnus vulgaris*, fed on an artificial nectar-like fluid diet of varying concentrations of sugar and protein. The MNR and TENL of the birds were similar and even slightly higher than allometrically expected values for birds of the starlings' mass (140% and 103%, respectively). This suggests that the low measured nitrogen requirements of nectar-feeding birds are not simply the result of their sugary and watery diets but a physiological adaptation to the low nitrogen input. We also measured the effect of water and protein intake on the nitrogenous waste form in the excreta and ureteral urine in European starlings. Neither high water intake nor low protein intake increased the fraction of nitrogen excreted as ammonia. Ammonia was excreted at consistently low levels by the starlings, and its concentration was significantly higher in ureteral urine than in excreta. We hypothesize that ureteral ammonia was reabsorbed in the lower intestine, indicating a postrenal modification of the urine.

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Introduction

In birds, the habit of feeding on nectar is accompanied by a variety of distinctive morphological and physiological characteristics. Nectar-feeding birds often have long and slender bills, hollow tongues, and lateral or reduced gizzards (Dresselberger 1932; Paton and Collin 1989). They also have unusual biochemical traits, such as exceedingly high disaccharidase activities and high rates of intestinal glucose transport, that allow them to assimilate nectar sugars efficiently and rapidly (Beuchat et al. 1979; Diamond et al. 1986; Karasov et al. 1986; Martínez del Rio 1990*a*, 1990*b*, 1994). Experiments on representative nectar-feeding bird species have revealed that their nitrogen requirements are extremely low (van Tets and Nicolson 2000; Brice and Grau 1991; Roxburgh and Pinshow 2000). These low nitrogen requirements are reasonably interpreted as adaptations to the low protein content of nectarivorous diets. Low nitrogen requirements, however, could be the results of both proximate and ultimate factors. The most important proximate factor is a fluid diet that is low in protein, fat, and fiber. Nectar is minimally abrasive, and assimilation of its contained sugars does not require secreting pancreatic enzymes and bile acids. Feeding on nectar presumably reduces the loss of metabolic fecal nitrogen (MFN), which is composed of nonabsorbed digestive enzymes, intestinal sloughed cells, and cell debris (Robbins 1993), and therefore we may not have to invoke evolutionary adaptation to explain the low nitrogen requirements of nectarivores.

So far, all nitrogen requirement experiments with nectarivorous birds have been measured on birds fed on watery, low-protein, and low-fiber diets (e.g., Brice and Grau 1991; Roxburgh and Pinshow 2000). In contrast, the nitrogen requirements of other birds have been studied when birds were fed diets that could potentially lead to higher MFN losses (reviewed by Klasing 1998; table 6.2). We conducted this study to determine if an omnivorous species, the European starling (*Sturnus vulgaris*), fed on a fluid diet similar to nectar, would also have low nitrogen requirements. We hypothesized that if the low nitrogen requirements of nectar-feeding birds are mainly the result of proximate factors, then starlings fed on a

nectar-like diet would have low nitrogen requirements similar to those of nectarivores.

The main nitrogen waste product in birds is uric acid and its salts (which we hereafter refer to as urates; Wright 1995). Urates are relatively insoluble and hence are excreted with little water. They are also comparatively nontoxic, but they are costly to synthesize (Klasing 1998). In contrast, ammonia is cheap to synthesize but fairly toxic and hence can only be used as a nitrogenous waste product by amphibious and aquatic animals with high rates of water excretion (Wright 1995). Preest and Beuchat (1997) suggested that it may be advantageous for birds ingesting large amounts of dilute, protein-poor nectar to shift from uricotely to ammonotely. Under these high-water-flux conditions, toxic ammonia might possibly be voided rapidly, and the costs of synthesizing urate can be reduced. Hence, we also studied the effect of protein and water intake on the concentrations of nitrogen products in excreta and in ureteral urine. We expected that starlings fed on fluid diets with low protein contents would increase the fraction of nitrogen excreted as ammonia and decrease the fraction that is voided as urates.

Material and Methods

Adult European starlings ($n = 10$) were held in captivity for 2 yr and maintained on a diet of chicken feed (Purina Starter Diet) and mealworms. The experiments were conducted in the same room where the birds were held, at the same ambient temperature (22° ± 2°C) and photoperiod (12L : 12D).

Experimental Protocol

During experiments, birds were fed synthetic fluid diets modified from those of Brice and Grau (1989). Diets contained glucose, fructose (starlings are sucrose intolerant; Martínez del Rio and Stevens 1989), NaCl, and casein acid hydrolysate as the nitrogen source (Sigma Chemical, St. Louis, MO). NaCl concentration was constant in all diets (9.07 mmol L^{-1}). We manipulated the protein intake of starlings by offering them diets that varied in both sugar and protein content (Table 1). Like many birds, starlings increase intake when fed on more dilute nectars. In our experiments, sugar concentration was the primary determinant of food intake. The birds did not appear to vary intake in response to variation in protein content (see "Results").

Twenty-four hours before experiments, birds were moved to individual experimental cages. During this period, the birds received the maintenance diet (Purina Layena: 16% crude protein, ≈8% fiber). Two hours before dark on the day before the experiment, food was removed from the cages to let the birds empty their gastrointestinal tract. Body mass was measured on the morning of the experiment and every 24 h thereafter. On the morning of the experiment (8 a.m.), galvanized metal pans containing 200 mL of white mineral oil were placed under the cages for collection of excreta. Fluid food was offered ad lib. in calibrated nonleaking glass feeding tubes. Evaporation through the feeders was negligible. Because the food consumption rate was high, feeders were refilled every 3–5 h. Pans were removed after 24 h, and excreta and mineral oil were collected into plastic bottles and frozen at −20°C for later analysis. New pans were placed under the cages for another 24-h period of collection. Ureteral urine samples were taken after birds had consumed the experimental diet for 24 h. Samples were collected by briefly inserting a closed-ended perforated cannula, made of polyethylene tubing (PE280), into the bird's cloaca (Goldstein and Braun 1986). All samples were stored frozen at −20°C for less than 7 d before analyses.

Table 1: Protein and sugar concentrations in the experimental diets of the European starlings

Trial, Diet	Sugar (%w/w)	Protein (g L^{-1})
Trial 1:		
1	10	0
2	20	1.2
3	10	4
4	10	1.2
5	20	4
Trial 2:		
1	5	8
2	10	12
3	5	12
4	5	10
5	5	2
6	5	4
7	5	6
8	5	10
9	5	4

Note. On the first trial, the experimental diet was given in duplicates, while on the second each bird received a different diet.

The experiment was conducted in two trials, using the same protocol but with different protein and sugar concentrations (Table 1). The birds had 2 wk to recover between trials. Food intake varied between trials as a result of the different sugar and protein concentrations; hence, we could not average the results of the same birds.

Sample Analysis

Excreta samples were thawed and separated from mineral oil by centrifugation (5,000 rpm for 3 min; Sorvall RC 5B Plus). Feather parts were removed with the oil, and an aliquot of each sample was taken for ammonia analysis. Because excreta contained a large amount of uric acid precipitates and solid material, we diluted the samples with 0.5 M LiOH (depending on the amount of uric acid in the sample, we used 1 : 1 or 1 : 10

dilutions). LiOH dissolves the urate precipitates and solubilizes ions trapped within urate crystals (Laverty and Wideman 1989; Roxburgh and Pinshow 2002). Samples were sonicated for further breakdown of the solid material and filtered through Whatman #1 filtered paper. All solids that accumulated on the filter were collected and dried at 50°C to constant mass. Clinical diagnostic kits (Sigma Chemical) were used to analyze uric acid (procedure 685), urea (procedure 535), ammonia (procedure 171-UV), and bile acids (procedure 450). Total soluble protein was assayed with the Bio-Rad protein assay kit II (catalog no. 500-0002, Bio-Rad Laboratories, Hercules, CA). Because the excreta samples were dissolved in lithium, we constructed standard curves using both lithium and deionized water. Adding lithium to our samples had no significant effect on the standard curves for urea, soluble protein, or uric acid (ANCOVA on intercepts and slopes, $P > 0.1$), but it had a significant effect on the determination of bile acids. Hence, we measured bile acid concentration in the aliquot that was taken before adding the lithium.

Nitrogen Requirements

Ureteral urine samples, excreta samples, and the dry solid material were analyzed for total nitrogen content in a CNH analyzer (Carlo Erba NA 1500). First, 15 μL of the liquid sample was transferred into tin capsules containing 5–10 mg of acid-washed Chromosorb W absorbant (Costech, Valencia, CA). Then, 5 mg of the solid samples were placed in tin capsules. Atropine was used as a standard for the elemental analysis. We used triplicates of the first 14 liquid samples. Because there were no significant differences among replicates (coefficient of variation [CV] = 5%), the rest of the samples were not replicated. For solid samples we used duplicates. At least 75% of the nitrogen in excreta was recovered by the chemical assays (Fig. 1). Nitrogen requirements and endogenous losses were determined by the regression of apparent nitrogen balance (nitrogen intake minus nitrogen excretion) on nitrogen intake (Brice and Grau 1991; Korine et al. 1996; Witmer 1998; Roxburgh and Pinshow 2000; Pryor et al. 2001).

Statistical Analysis

ANCOVA was used to test the relationship between protein and sugar concentration on intake response. Least squares linear regression was used to test for correlation between excretion of all forms of nitrogen waste and water or nitrogen intake. Paired *t*-tests were used to compare body mass differences between the beginning and the end of the experiment and to compare the concentrations of nitrogen waste forms in ureteral urine and excreta. Values are reported as means ± SE. Significance was accepted at $P < 0.05$.

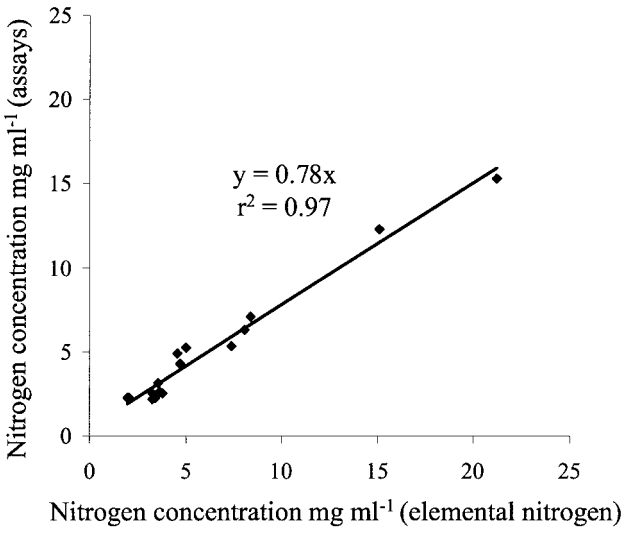

Figure 1. Correlation between the concentrations of nitrogen in the excreta of European starlings recovered by the different assays (urate, ammonia, urea, soluble proteins, and bile acids) and the concentration of nitrogen in the excreta recovered by elemental analysis. The concentration of assayed nitrogen from the excreta samples was significantly positively correlated with the nitrogen recovered by elemental analysis: $y = 0.78x$, $r^2 = 0.97$. This indicates that, on average, 80% of the nitrogen in excreta was detected by the assays and consisted of urate, ammonia, urea, soluble proteins, and bile acids. Other nitrogenous compounds, such as nitrogen oxides, contributed the remaining 20%.

Results

Food Consumption

Starlings maintained a constant body mass during the second trial only (paired *t*-test of bird body mass before and after trial, $t_8 = 0.48$, $P > 0.05$), while they lost body mass during the first ($t_8 = 8.3$, $P < 0.05$). Starlings exhibited an intake response similar to that of nectarivorous birds (Martínez del Rio et al. 2001). They consumed less solution at higher than at lower sugar concentrations (Spearman rank correlation coefficient = -0.60, $P < 0.01$, $n = 19$). The relationship between volumetric intake (I) and sugar concentration (C) was described by a power function ($\log I = 2.8 + 0.52 \log C$, $r^2 = 0.58$, $n = 28$, $P < 0.01$). At our experimental concentrations, nitrogen content did not have an effect on intake ($\text{ANCOVA}_{\text{nitrogen}}$, $F_{1,18} = 0.66$, $P > 0.4$, when diet nitrogen level was added to a food intake model as a covariate). Starlings consumed on average 63.3 ± 4.1 mL d^{-1} of fluid food and excreted 18% ± 2.1% of their intake. Starlings consumed an average of 6.21 ± 0.4 g d^{-1} sugar. Assuming 16.5 kJ g^{-1} of sugar, they consumed 102.5 ± 6 kJ d^{-1}. As a result of the simultaneous manipulation of sugar and protein concentrations, starlings ingested from 0 to 158 mg d^{-1} of nitrogen.

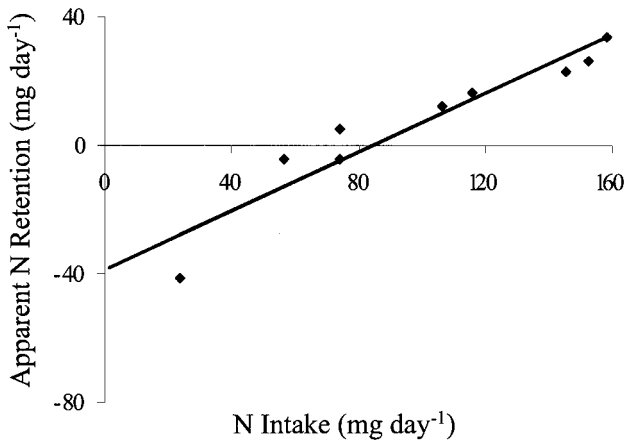

Figure 2. Dependence of apparent nitrogen retention on nitrogen intake in European starlings consuming a fluid diet. Apparent nitrogen retention increased significantly with nitrogen intake ($y = 0.46x - 38.66$, $r^2 = 0.90$). Total endogenous nitrogen losses (TENL) and maintenance nitrogen requirements (MNR) were calculated from the *y*- and *x*-intercepts of a least squares linear regression model, respectively. MNR and TENL values are reported in the text.

Nitrogen Requirements

Nitrogen requirements were calculated on the second experiment when the birds maintained a constant body mass. Apparent nitrogen retention (intake minus excretion) increased significantly with nitrogen intake ($y = 0.46x - 38.66$ mg d^{-1}, $r^2 = 0.92$, $n = 9$, $P < 0.0001$). Nitrogen content of dry matter excreted by the birds was added to the excreted nitrogen and represented on average 4.5% ± 0.65% of all excreted nitrogen. We estimated total endogenous nitrogen losses (TENL) and maintenance nitrogen requirements (MNR) from the *y*- and *x*-intercepts of a least squares linear regression model relating apparent nitrogen retention and intake (Brice and Grau 1991; Korine et al. 1996; Witmer 1998; Delorme and Thomas 1999; van Tets and Hulbert 1999; Roxburgh and Pinshow 2000). The calculated values were MNR = 84.04 ± 7.3 mg N d^{-1} and TENL = 38.7 ± 6.3 mg N d^{-1} (Fig. 2). The estimated MNR and TENL levels were 140.7% and 103.2% of those expected for a bird of the starling's body mass from the allometric equations of Robbins (1993; 59.7 and 37.5 mg d^{-1}, respectively). This result contradicts our first prediction. Simply ingesting a fluid sugary solution does not lead to low nitrogen requirements.

Nitrogen Intake versus Excreted Nitrogenous Waste Forms

Birds excreted nitrogen in detectable quantities as uric acid (urate), ammonia, urea, soluble proteins, and bile acids (which constituted less than 0.1% of the assayed excreted nitrogen). European starlings excreted nitrogen predominantly as uric acid (more than 60% of the nitrogenous compounds excreted), but they also excreted urea (less than 20%), ammonia (less than 20%), and protein (less than 10%; Fig. 3*a*). Only the rate of nitrogen excreted as urate increased significantly ($P < 0.05$) with daily protein intake ($r^2 = 0.34$, $n = 19$; Fig. 3*b*); the rates of nitrogen excreted as urea (4.43 ± 0.34 mg d^{-1}), ammonia (6.5 ± 0.71 mg d^{-1}), and soluble protein (3.76 ± 0.42 mg d^{-1}) remained unchanged with protein intake ($r^2 = 0.19$, 0.005, and 0.05, respectively, $n = 19$, $P > 0.05$). The percentage of nitrogen excreted as urate increased significantly with protein intake ($r^2 = 0.34$, $n = 19$, $P < 0.01$), whereas the percentage as urea decreased significantly ($r^2 = 0.34$, $n = 19$, $P < 0.01$). The percentages of ammonia and soluble proteins did not change with protein intake ($r^2 = 0.05$ and 0.05, respectively, $n = 19$, $P > 0.05$). There were no significant correlations between water intake and the rate or percentage of any of the nitrogenous products in excreta. When we included both water and protein

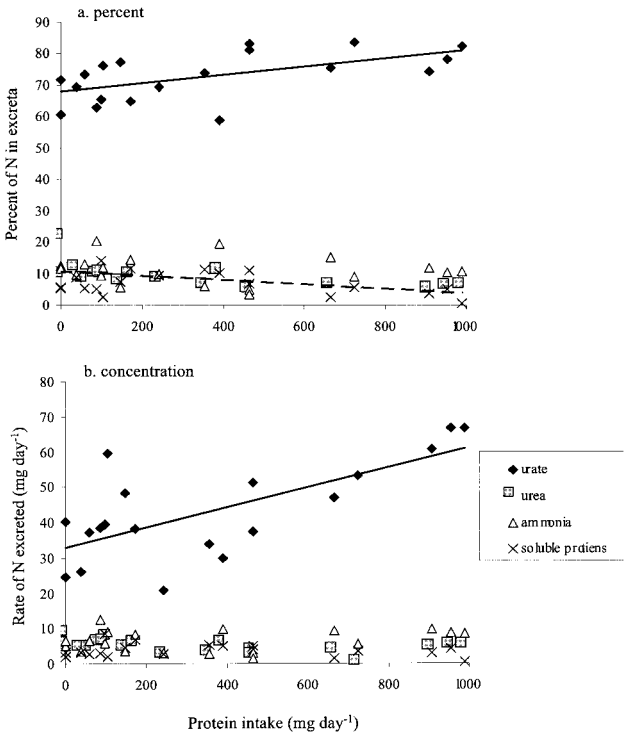

Figure 3. *a*, Correlation between the percentage of nitrogen excreted as urate, ammonia, urea, and soluble protein and protein intake in European starlings. Urate comprised, on average, 70% of the assayed nitrogen, and its percentage increased significantly with protein intake ($y = 0.013x + 68$, $r^2 = 0.34$, $P < 0.01$), whereas the percentage of urea significantly decreased with protein intake ($y = -0.007x + 10.7$, $r^2 = 0.36$, $P < 0.01$). The percentage of nitrogen excreted as ammonia and soluble proteins did not change with protein intake. *b*, Correlation between the rates of nitrogen excreted as urate, ammonia, urea, and soluble-protein and protein intake. The rate of nitrogen excreted as urate from total assayed nitrogen in excreta increased significantly with protein intake ($y = 0.03x + 32.8$, $r^2 = 0.49$, $P < 0.05$), whereas those of ammonia, urea, and soluble proteins remained constant.

intake in multiple regressions with urate or ammonia concentration as response variables, we found that protein intake had a significant effect but water intake did not.

Comparison between Excreta and Ureteral Urine

The concentrations of urate and soluble protein did not differ between the excreta and ureteral urine (paired t-test, $P > 0.05$). However, the concentration of ammonia was significantly higher in ureteral urine than in excreta (paired t-test, $t_{14} = 7.07$, $P < 0.0001$; Fig. 4*a*), whereas the concentration of urea was significantly lower in ureteral urine than in excreta (paired t-test, $t_{16} = -4.98$, $P < 0.005$; Fig. 4*b*).

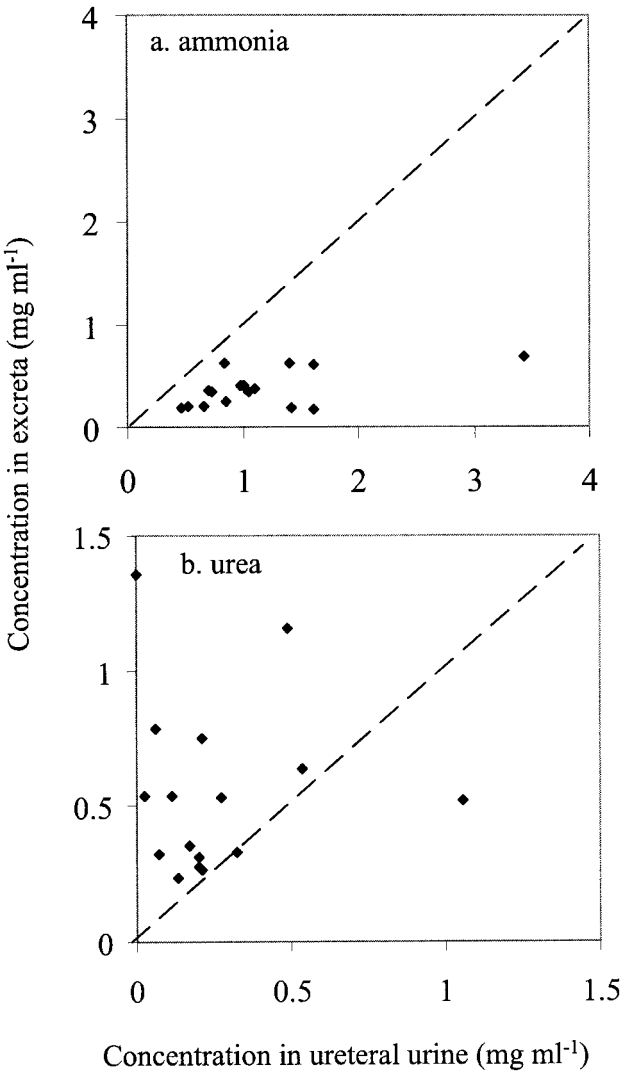

Figure 4. *a*, In European starlings, the concentration of ammonia was significantly higher in ureteral urine than in excreta, indicating that ammonia might have been absorbed in the lower guts of the birds. *b*, The concentration of urea was significantly higher in excreta. Urea is a by-product of microbial degradation of urate, but the concentration of urate did not differ between ureteral urine and excreta, suggesting low rates of urate microbial degradation.

Discussion

A fluid diet is not, in itself, the reason for the low nitrogen requirements of nectar-feeding birds. Contrary to our prediction, our results indicate that the fluid diet itself did not affect the nitrogen requirements of the European starlings. The nitrogen requirements of starlings feeding on nectar-like fluid diets with exceedingly low nitrogen contents were not very different from those expected from allometric predictions. The estimated MNR and TENL values were 140% and 103% of those expected for a bird of the starling's body mass (Robbins 1993). In contrast, those of hummingbirds were only 15%–30% of their respective predicted values (reviewed by McWhorter et al. 2003). Thus, the primary determinant of the low nitrogen requirements and endogenous nitrogen loss of nectar-feeding animals appears to be an evolutionary adaptation to a low-protein diet. The mechanistic bases for such an adaptation remain mysterious and include low rates of endogenous protein turnover and conservation of excreted nitrogen (reviewed by McWhorter et al. 2003). Korine et al. (1996) speculated that high carbohydrate intake may stimulate insulin secretion. Because insulin inhibits gluconeogenesis (Fukagawa et al. 1985; Florini 1987), it may reduce the use of amino acids and hence minimize protein turnover.

Nitrogen conservation has been documented in a variety of Galliformes, such as willow ptarmigan, Gambel's quail, and domestic chickens (Mortensen and Tindall 1981; Campbell and Braun 1986; Son and Karasawa 2000). These birds have large cecae populated by anaerobic microorganisms (Mead 1997; Clench 1999), some of which use uric acid as a major source of carbon and energy (Mead 1997). Thus, urates enter the cecae by retroperistalsis are degraded there. Karasawa et al. (1988) proposed that the ammonia produced from the fermentation of uric acid is absorbed by Galliformes for synthetic purposes.

Postrenal modification of urine in starlings. Although nitrogen conservation mediated by microorganisms has not been documented in birds with rudimentary cecae (like passerines) or without cecae (like hummingbirds), it may still occur. Roxburgh and Pinshow (2002) observed that when maintained on low-protein diets, seven out of 52 tested Palestine sunbirds excreted more nitrogen as ammonia than as uric acid. This ammonotely was only "apparent," because the concentration of urate and urea dropped when dietary nitrogen was low, whereas the absolute quantity of ammonia did not. They explained this apparent ammonotely as a result of microbial postrenal modification of urine. They speculated that microbes degraded uric acid and generated ammonia. Preest et al. (2003) documented degradation of potassium urate (but, curiously, not of sodium urate) by microbes extracted from the intestine

of Anna's hummingbirds. European starlings may also possess postrenal urine modification mechanisms. In this study, ammonia concentration was significantly higher in ureteral urine than in excreta, whereas urea concentration was significantly lower. We hypothesize that ammonia from urine was reabsorbed in the lower intestine. The significantly higher concentration of urea in excreta than in ureteral urine is difficult to explain. Because urea is a by-product of microbial degradation of urate, it is tempting to speculate that microbe-mediated urate catabolism was responsible for this increase. However, the concentration of urate did not differ between ureteral urine and excreta, suggesting low rates of urate microbial degradation.

Low protein and high water intake do not induce ammonotely in starlings. Like most "typical" birds, starlings excreted most of their nitrogen as uric acid. Even when they consumed large amounts of water (from 35% to 140% of their body mass per day), they did not become ammonotelic under any condition and always excreted more nitrogen as uric acid than as ammonia. Neither high water intake nor low protein intake increased the fraction of nitrogen excreted as ammonia. Ammonia was excreted at consistently low levels by starlings.

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