

## Can birds be ammonotelic? Nitrogen balance and excretion in two frugivores

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### Summary

We measured minimal nitrogen requirements (MNR), total endogenous nitrogen loss (TENL) and the effect of protein and water intake on the nitrogenous waste composition in two frugivorous bird species: yellow-vented bulbuls *Pycnonotus xanthopygos* and Tristram's grackles *Onychognathus tristrami*. The nitrogen requirements of both species were much lower than expected for their body mass. The two species differed in the composition of the nitrogenous waste that they produced. The grackles were uricotelic, and the chemical composition of their nitrogenous waste products was relatively independent of water and protein intake. In contrast, the bulbuls were 'apparently ammonotelic'.

Their ammonotelic was related to low protein intake and high water flux, and was the result of post-renal urine modification. We suggest two non-exclusive mechanisms for the post-renal modification of urine in these birds: bacterial catabolism of uric acid and reabsorption of uric acid in the hindgut. As uric acid functions both as a nitrogenous waste product and as an antioxidant, birds might benefit from its reabsorption.

Key words: yellow-vented bulbul, *Pycnonotus xanthopygos*, Tristram's grackle, *Onychognathus tristrami*, nitrogen balance, nitrogen requirements, uric acid, ammonia, frugivores.

### Introduction

Birds are believed to be primarily uricotelic (Wright, 1995). Uric acid is a relatively non-toxic nitrogen end product. It is relatively insoluble and hence excreted with little water. Uricotelic, however, is costly. More energy is needed to excrete a unit of waste nitrogen as uric acid than as urea or ammonia (Wright, 1995; Klasing, 1998). In contrast to uric acid, ammonia is highly soluble, cheap to synthesize, but fairly toxic (Wright, 1995). It can only be used as a nitrogenous waste by animals with high rates of water turnover that permit almost continuous elimination, such as in aquatic animals (Wright, 1995).

Preest and Beuchat (1997) suggested that it might be advantageous for birds that ingest large amounts of dilute, protein-poor nectar to shift from uricotelic to ammonotelic. Thus, ammonia can be voided rapidly, and the costs of synthesizing urates can be reduced. Preest and Beuchat (1997) called the shift from uricotelic to ammonotelic in hummingbirds 'facultative ammonotelic'. In their study on Anna's hummingbird *Calypte anna*, Preest and Beuchat concluded that these birds were facultatively ammonotelic. At low ambient temperatures and high water intakes, Anna's hummingbirds excreted more than 50% of their total nitrogen excretion as ammonia (i.e. they became ammonotelic), whereas at higher ambient temperatures and lower food intake they were uricotelic (Preest and Beuchat, 1997). The hypothesis of Preest

and Beuchat (1997) proposes that high energy demands and high water fluxes favor ammonotelic.

McWhorter et al. (2003) challenged the generality of Preest and Beuchat's results (Preest and Beuchat, 1997). They fed black-chinned hummingbirds *Archilochus alexandri*, magnificent hummingbirds *Eugenes flavifrons* and blue-throated hummingbirds *Lampornis clemenciae* dilute, protein-poor diets, but found that neither of these species became ammonotelic. The hummingbirds remained uricotelic even when they fed on the most dilute food and when they ingested and excreted prodigious amounts of water (>3 times their body mass per day). Urate constituted over 60% of all nitrogenous waste in all species and water intake had no effect on the proportion of nitrogen excreted as ammonia.

To further cast doubt on the generality of facultative ammonotelic in nectarivorous birds, Roxburgh and Pinshow (2002) found that Palestine sunbirds *Nectarinia osea* were generally uricotelic. However, in seven out of 52 tests, *N. osea* excreted more nitrogen as ammonia than as uric acid. Because the fraction of nitrogen excreted as ammonia never exceeded 50%, these birds were not ammonotelic. However, they were clearly more ammonotelic than uricotelic. Roxburgh and Pinshow (2002) noted that in birds with high water intake, the concentration of urate was higher in ureteral urine than in excreta. They argued that ammonotelic in Palestine sunbirds

was only 'apparent' as it was not a result of excessive excretion of ammonia, but rather the result of a reduction in excreted urate resulting from post-renal modification of urine.

Although apparent ammonotely has not yet been observed in non-nectarivorous species it might not be a unique feature of nectarivorous birds (Roxburgh and Pinshow, 2002). We hypothesized that apparent ammonotely is related to low nitrogen intake and high water fluxes. We only tested the second component of Preest and Bucheaut's hypothesis (Preest and Bucheaut, 1997), but added nitrogen intake as an interacting factor. Our experiments did not address the effect of increased energy demands on the composition of excreted nitrogenous products. Specifically, we predicted that birds with low nitrogen intake recover some of the uric acid in the lower gastrointestinal tract. This reduction leads to an apparent increase in the fraction of nitrogen excreted as ammonia. Because fruit-eating birds appear to have unusually low nitrogen intake (Witmer, 1998; Pryor et al., 2001), we hypothesized that we would find apparent ammonotely in two frugivorous birds: the yellow-vented bulbul *Pycnonotus xanthopygos* and the Tristram's grackle *Onychognathus tristrami*. To test this idea we manipulated protein and water intake by simultaneously varying the protein and water contents of the diet and by measuring their effects on the composition of nitrogenous waste in excreta and in ureteral urine. Our experimental protocols allowed us to also measure minimal nitrogen requirements (MNR) and total endogenous nitrogen loss (TENL) in two desert frugivorous birds.

## Materials and methods

### Study species

Yellow-vented bulbuls *Pycnonotus xanthopygos* Pycnonotidae (body mass  $M_b$ :  $36.5 \pm 0.6$  g,  $N=10$ ) breed in Israel, western Jordan, Sinai and along western and southern Arabia (Shirihai, 1996). In Israel they consume many wild and cultivated fruit species (Izhaki and Safriel, 1985; Izhaki et al., 1991; Barnea et al., 1991) and may be the most prominent frugivorous species in the Israeli avifauna. Tristram's grackles *Onychognathus tristrami* Sturnidae ( $M_b$ :  $117.4 \pm 1.5$  g,  $N=9$ ) are omnivores of Saharo-Arabian origin. They feed primarily on fruits and small invertebrates (Cramp and Perrins, 1994).

### Care and maintenance

#### Tristram's grackle

Seven birds were mist-netted under license from the Israel Nature and National Parks Authority in the Dead Sea region one month before the experiments. Two other birds were long-term captives on the campus of Haifa University at 'Oranim', Kyriat Tivon, where all birds were kept and where all experiments were conducted. Birds were held in a large open outdoor cage and maintained on fruits (apples, grapes, melons) and chopped boiled eggs.

#### Yellow-vented bulbuls

Ten birds were mist-netted on the 'Oranim' campus, Kyriat

Tivon, and at Sde-Yaakov, northern Israel (under license from the Israel Nature and National Parks Authority). Birds were maintained in separate cages (60 cm×30 cm×60 cm) in an outdoor shed. Maintenance food contained fruits (apples, grapes, melons), vegetables (tomatoes, cucumbers) and chopped boiled eggs. In all experiments birds were housed in individual cages (40 cm×30 cm×30 cm) in a room maintained at  $20 \pm 2^\circ\text{C}$  with a photoperiod of 12 h:12 h light:dark.

### Intake response experiment

On the day before experiments, birds were transferred to the experimental cages (bulbuls  $N=10$ , grackles  $N=9$ ) and offered a fluid diet in plastic feeders containing different concentrations of casein acid hydrolysate (Sigma Chemical, St Louis, MO, USA), and a 1:1 mixture of glucose and fructose. Each bird was fed a solution containing one of the following sugar concentrations: 5, 10, 15, 20 or 25% (g sugar per 100 g of solution). The sugar solutions also contained one of two possible casein hydrolysate concentrations. Bulbuls were fed 2, and 6 g  $\text{l}^{-1}$ , and grackles were fed 1.2 and 6 g  $\text{l}^{-1}$ . After 8 h, the amount of solution consumed was measured. The variation in protein intake that we observed in these experiments allowed us to control the protein intake in subsequent experiments.

### Nitrogen requirements and composition of urine and excreta

Experiments lasted 3 days. During experiments birds were offered synthetic fluid diets, modified from those of Brice and Grau (1989). These diets varied in sugar and protein concentrations (Table 1). NaCl concentration was constant in all diets (9.07 mmol  $\text{l}^{-1}$ ). Birds were transferred to individual experimental cages 3 days before experiments commenced. During this period, the birds received the maintenance diet. On the day prior to an experiment food was removed from the cages 2 h before dark. We measured body mass on the first morning of the experiment and every 24 h thereafter. Plastic pans containing 200 ml of mineral oil were placed at 08:00 h under the cages for collection of excreta. Pans were removed after 24 h and the excreta and mineral oil were collected into plastic bottles and frozen at  $-20^\circ\text{C}$  for later analysis. New pans were placed under the cages for another 24 h collection period. Only the 24 and 72 h (first and third day, respectively) collections were analyzed. Two birds were randomly assigned to each of the five diets. Food was offered *ad libitum* in plastic feeders. Fluid consumption was measured every 24 h.

### Collection of ureteral urine and plasma

Ureteral urine samples were collected at 08:00 h after the birds had consumed the experimental diet for 24 h and 72 h. Samples were obtained by briefly inserting a closed-ended perforated cannula (Goldstein and Braun, 1986), custom-made of polyethylene tubing (Tristram's grackle PE300, yellow-vented bulbuls PE200; Intramedic, MD, USA) into the bird's cloaca. The samples were then kept at  $-20^\circ\text{C}$  for later analysis. We collected blood ( $\sim 100$   $\mu\text{l}$ ) in heparinized microhematocrit tubes by puncturing the brachial vein with a 28-gauge needle.

Table 1. Protein and sugar concentrations in the experimental diets of yellow-vented bulbuls and Tristram's grackles

Birds	Diet	Sugar (%w/w)	Protein (g l <sup>-1</sup> )
Yellow-vented bulbul	1	10	0
	2	20	2
	3	10	3
	4	10	6
	5	10	8
Tristram's grackle	1	20	0
	2	20	1.7
	3	20	3.3
	4	20	7.9
	5	20	15

Plasma was separated from cells after centrifugation (microhematocrit centrifuge, International Equipment CL A4922X-1, Needham, MA, USA) for 3 min.

#### Sample analysis

Excreta samples were thawed and separated from the mineral oil by centrifugation (5000 r.p.m. for 3 min, Sorvall RC 5B Plus, Asheville, NC, USA). Feather parts were removed

with the oil and an aliquot sample of each sample was taken for ammonia, urea and soluble protein analysis. The samples were dissolved in 0.5 mol l<sup>-1</sup> LiOH. LiOH dissolves the urate precipitates and any trapped ions (Roxburgh and Pinshow, 2002). Clinical diagnostic kits (Sigma Chemical, St Louis, MO, USA) were used to analyze uric acid (procedure no. 685) and urea (procedure no. 535). Ammonia was assayed using the Roche ammonia UV-test (catalog no. 1-112-732, Roche, Indianapolis, IN, USA). Total soluble protein was assayed with the Bio-Rad Protein Assay Kit II (catalog no. 500-0002, Bio-Rad Laboratories, Hercules, CA, USA). Because the uric acid in excreta samples was dissolved in lithium, we constructed standard curves using both lithium and de-ionized water. Adding lithium to our samples had no significant effect on the standard curves of uric acid (ANCOVA on intercepts and slopes,  $P>0.1$ ).

#### Nitrogen requirements

Nitrogen requirements and endogenous losses were determined by the regression of the apparent nitrogen balance (nitrogen intake minus nitrogen excretion) on nitrogen intake (Brice and Grau, 1990; Korine et al., 1996; Witmer, 1998; Roxburgh and Pinshow, 2000; Pryor et al., 2001), using the total nitrogen recovered from the different assays.

#### Statistical analysis

We used linear models to determine the effect of protein and sugar concentrations on the intake response and to compare the nitrogen requirements between the experimental days. ANCOVA was also used to compare plasma urate concentrations between the species. Least-squares linear regression was used to test for correlations between excretions of all forms of nitrogenous waste and the water or nitrogen intake. Paired *t*-tests were used to determine the significance of changes in  $M_b$  over the experimental period and to compare the concentrations of nitrogenous waste forms in ureteral urine and in excreta. A bird was considered ammonotelic if more than 50% of its total nitrogen excretion was excreted as ammonia. Thus, we used a one sample *t*-test to test for ammonotely in both species. The exponent of the power relationship between volumetric intake and sugar concentration in food should equal  $-1$  if birds compensate perfectly for energy dilution by increasing intake (Martínez del Rio et al., 2001). Thus, we used one sample *t*-test to test whether the exponent of the power relationship differed from  $-1$ . Multiple regressions were used to determine the effect of water and protein

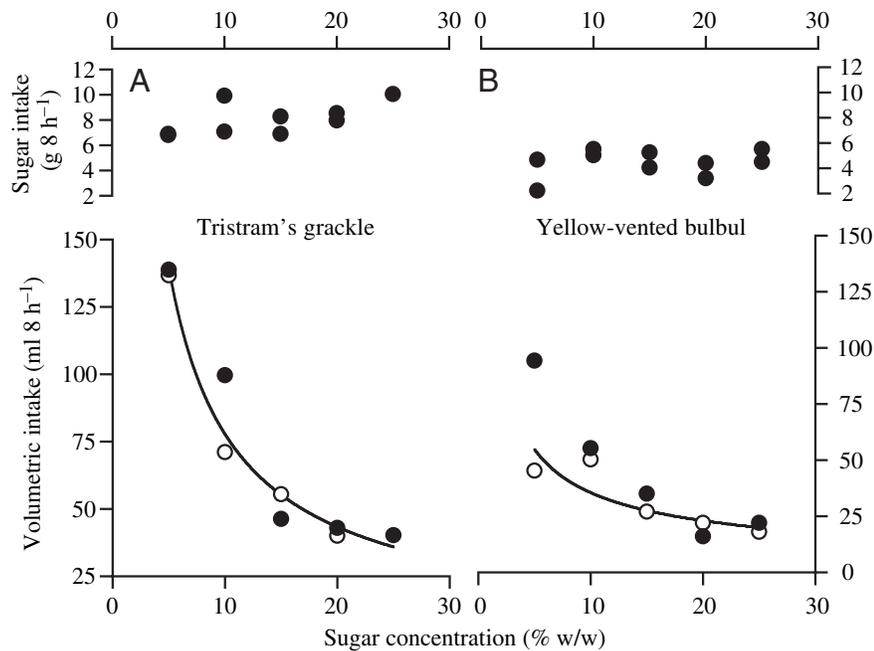
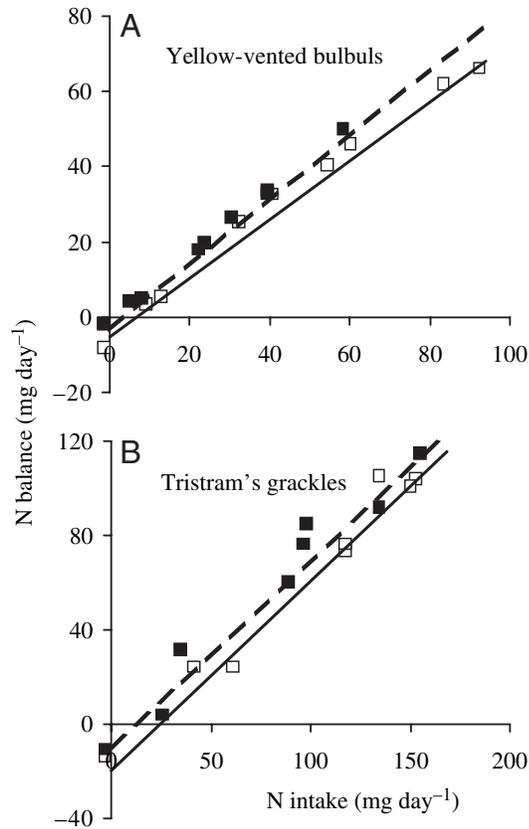


Fig. 1. The effect of sugar concentration on sugar intake. Tristram's grackles (A) and Yellow-vented bulbuls (B) exhibit a typical intake response: their sugar intake (top) was independent of sugar concentration. They decrease volume of intake (D) (bottom) as the sugar concentration in food (C) increases (Martínez del Rio et al., 2001). The intake response of both species is well described by power functions ( $I=543C^{-0.85}$ ,  $r^2=0.94$  and  $I=285C^{-0.85}$ ,  $r^2=0.79$  for Tristram's grackles and yellow-vented bulbuls, respectively). Closed circles, low protein concentration in diet; open circles, high protein concentration in diet (see text for the concentration values). Because there were no significant effects of protein content on volumetric intake the power functions were fitted to pooled data.



intake and of experimental day on the percentage of nitrogen excreted as ammonia and as uric acid. Values are reported as means  $\pm$  S.E.M.

## Results

### Intake response

Because the relationships between volumetric intake and sugar concentration are well described by power functions

Fig. 2. Apparent nitrogen retention increased significantly with nitrogen intake in both species. Minimal nitrogen requirements (MNR) and total endogenous nitrogen losses (TENL) were determined using the  $x$  and  $y$  intercepts of the least-squares linear regression, respectively. Yellow-vented bulbuls:  $y=0.78x-5.4$ ,  $r^2=0.99$ ,  $F_{1,8}=797$ ,  $P<0.0001$ ;  $y=0.86x-3.4$ ,  $r^2=0.99$ ,  $F_{1,8}=7208$ ,  $P<0.0001$ , first and third day, respectively. Tristram's grackle:  $y=0.8x-19.8$ ,  $r^2=0.98$ ,  $F_{1,6}=277.6$ ,  $P<0.0001$ ;  $y=0.8x-11$ ,  $r^2=0.97$ ,  $F_{1,6}=231.2$ ,  $P<0.0001$ , first and third day, respectively. TENL in both species decreased significantly between the first and third day of experiment. In yellow-vented bulbuls, but not in Tristram's grackles, MNR also decreased on the third day. MNR and TENL values are presented in Table 2. Open squares and solid lines represent measurements on day 1 and closed squares and broken lines represent measurements on day 3.

(Martínez del Río et al., 2001), we analyzed intake response data on log-transformed variables. In both species, volumetric food intake (in ml  $8\text{ h}^{-1}$ ) decreased significantly with increased sugar concentration (% w/w,  $F_{1,9}=30.8$  and  $F_{1,8}=112.6$ ,  $P<0.001$  for bulbuls and grackles, respectively; Fig. 1). Protein content of the diet had no effect on the volumetric intake ( $F_{1,9}=1.3$  and  $F_{1,8}=0.9$ ,  $P>0.2$  for bulbuls and grackles, respectively). The exponent of the power functions relating volumetric intake to sugar concentration of the diet did not differ significantly from  $-1$  ( $t_9=1.1$ , and  $t_8=1.7$ ,  $P>0.1$ , for bulbuls and grackles, respectively). Thus, sugar intake was independent of sugar concentration (Fig. 1). Bulbuls consumed  $0.29\pm 0.02$  g of sugar per unit  $M_b^{0.75}$  per 8 h and grackles consumed  $0.23\pm 0.04$  g per unit  $M_b^{0.75}$  per 8 h. Protein intake was proportional to volumetric intake and increased with decreasing sugar concentration.

### Nitrogen requirements

Both species maintained constant  $M_b$  during experiments (paired  $t$ -test before and after experiments: bulbuls,  $t_9=-1.7$ ,  $P>0.1$ ; grackles,  $t_8=1.1$ ,  $P>0.1$ ). During experiments, nitrogen

Table 2. Minimal nitrogen requirements and total endogenous nitrogen loss of yellow-vented bulbuls and Tristram's grackles

Bird	Day	Body mass (g)	Measured (mg day <sup>-1</sup> )	Expected (mg day <sup>-1</sup> )*	Measured/expected
Grackle	MNR	1	24.7	85.5	0.29
		3	13.8		0.16
	TENL	1	19.9	53.7	0.37
		3	11		0.21
Bulbul	MNR	1	8.16	36.1	0.23
		3	4.3		0.12
	TENL	1	6.2	22.7	0.27
		3	3.7		0.16

MNR, minimal nitrogen requirements; TENL, total endogenous nitrogen loss.

$N=20$  (yellow-vented bulbuls);  $N=18$  (Tristram's grackles).

\*Calculated from the allometric equations of Robbins (1993).

intake ranged from 0 to 1.1 g per day in grackles and from 0 to 0.58 g per day in bulbuls (assuming 1 g protein,  $\approx 0.16$  g nitrogen). MNR and TENL were calculated based on the nitrogen recovered from chemical assays. In a previous study (Tsahar et al., 2005), we recovered over 80% of the elemental nitrogen in chemical assays. Thus, we assumed that using the assayed nitrogen underestimates nitrogen requirements only slightly.

In both species, a significant positive correlation was found between nitrogen balance and nitrogen intake (Fig. 2). The estimated MNR and TENL for both species on the first and third days and their expected values (based on Robbins, 1993) are summarized in Table 2. In both species MNR and TENL after 72 h were lower than after 24 h of feeding on the experimental diets. MNR decreased by 44% in grackles and by 47% in bulbuls, and TENL decreased by 45% in grackles and

by 40% in bulbuls. In grackles, only the intercept changed significantly between days while the slope did not ( $F_{(\text{slope})1,14}=0.004$ ,  $P>0.9$ ;  $F_{(\text{intercept})1,14}=-3.81$ ,  $P<0.002$ ), whereas in bulbuls both differed between days ( $F_{(\text{slopes})1,16}=4.65$ ,  $P<0.05$ ;  $F_{(\text{intercepts})1,16}=-6.04$ ,  $P<0.0001$ ).

#### Factors affecting the chemical composition of the nitrogenous waste

We used multiple regressions to assess the effect of water and protein intake and experimental day on the percentage of nitrogen excreted as either ammonia or urate. We included two data points for each of the nine grackles or ten bulbuls in these regressions and hence our analysis can be considered pseudoreplicated. When we included bird identity as a factor in the linear model, its contribution was not significant. Hence we dropped individual identity from the model. In bulbuls, the percentage of total nitrogen excreted as ammonia did not depend on day ( $F_{1,16}=0.03$ ,  $P=0.85$ ), was negatively correlated with protein intake ( $F_{1,16}=5.9$ ,  $P=0.03$ ), and was positively correlated with water intake ( $F_{1,16}=18.7$ ,  $P=0.0005$ ; Fig. 3, Table 3). The percentage of nitrogen excreted as uric acid was tightly and negatively related to the percentage of nitrogen excreted as ammonia ( $\% \text{uric acid} \approx 100 - \% \text{ammonia}$ ). Thus, in bulbuls the proportion of nitrogen excreted as uric acid did not depend on experimental day ( $F_{1,16}=0.13$ ,  $P=0.73$ ), but was positively related to protein intake ( $F_{1,16}=8.68$ ,  $P=0.009$ ) and negatively related to water intake ( $F_{1,16}=15.69$ ,  $P=0.001$ ). In grackles, the percentage of nitrogen excreted as either ammonia or urate did not depend on day ( $F_{1,14}=4.29$ ,  $P=0.06$ ;  $F_{1,14}=1.72$ ,  $P=0.21$ , respectively), protein intake ( $F_{1,14}=1.55$ ,  $P=0.23$ ;  $F_{1,14}=3.17$ ,  $P=0.097$ , respectively), or water intake ( $F_{1,14}=1.21$ ,  $P=0.29$ ;  $F_{1,14}=1.07$ ,  $P=0.32$ , respectively). Protein and urea represented a very small percentage of all nitrogen excreted in both species (Table 4). In both species water intake decreased significantly between the first and third experimental days (Table 4). In bulbuls the percentage of nitrogen excreted as urate increased significantly between the first and third day and the percentage of nitrogen excreted as ammonia decreased significantly. In Tristram's grackles, the percentage of nitrogen excreted as urate and ammonia did not change significantly between experimental days.

Taken together, we can conclude that bulbuls were ammonotelic (Fig. 4). The percentage of ammonia in their excreta did not differ significantly from 50% (Table 4, one sample  $t_{19}=0.13$ ,  $P>0.5$ ). In contrast, grackles were significantly uricotelic ( $t_{19}=7.3$ ,  $P<0.0001$ ; Table 4, Fig. 4). However, pooling all individuals may not be appropriate for bulbuls. The results presented in Fig. 4A suggest that the percentage of nitrogen excreted as ammonia increased significantly with water intake. About 80% of all bulbuls that ingested more than 60 ml of water per day were ammonotelic. In contrast, all but one of the grackles were uricotelic under all conditions.

#### Comparison between excreta and ureteral urine

In bulbuls, the concentrations of urate and soluble proteins were higher in ureteral urine than in excreta (Table 5).

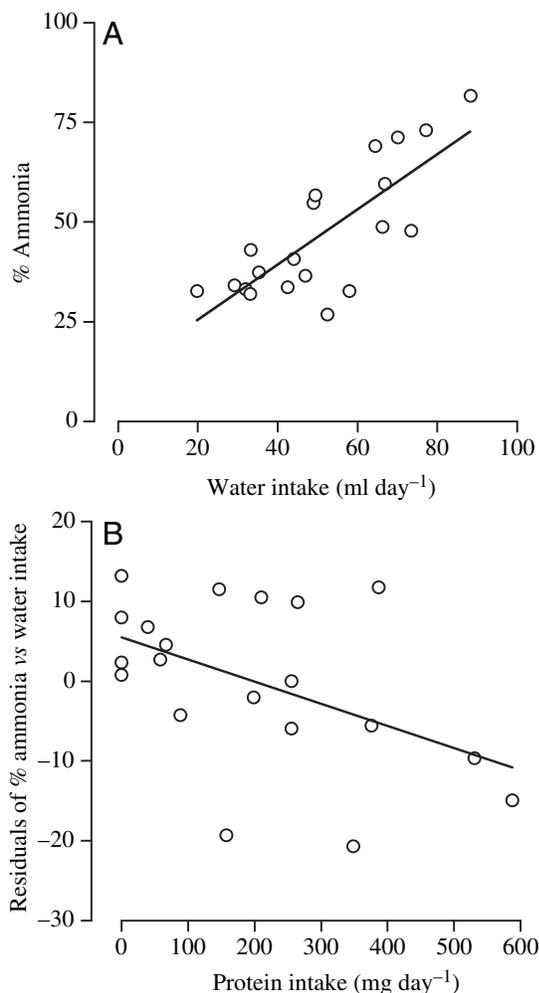


Fig. 3. Effect of water and nitrogen intake on excreta. In yellow-vented bulbuls, both water and protein intake had a significant effect on the proportion of nitrogen excreted as ammonia. The proportion of nitrogen excreted as ammonia was positively correlated with water intake (A:  $y=0.6x+18.4$ ,  $r^2=0.54$ ,  $F_{1,18}=21.3$ ,  $P=0.0002$ ). The residuals of this relationship were negatively correlated with protein intake (B:  $y=-0.03x+5.5$ ,  $r^2=0.23$ ,  $F_{1,18}=5.4$ ,  $P=0.03$ ).

Table 3. Multiple regression analysis of the factors affecting the percentage of nitrogen excreted as ammonia and as urate, in the yellow-vented bulbul\*

		Intercept	Water intake	Protein intake	Day
Ammonia	Regression coefficient	17.51	0.74	-0.03	-0.56
	<i>t</i> value		4.3	-2.4	-0.19
	<i>P</i>	0.06	0.0005	0.026	0.8
Urate	Regression coefficient	69.5	-0.75	0.04	1.17
	<i>t</i> value		-3.96	2.95	0.35
	<i>P</i>	<0.0001	0.001	0.009	0.7

\*N=20.

Water intake had a positive effect on ammonia excretion and a negative effect on the excretion of urate. Protein intake had the opposite effect on these two metabolites.

Table 4. The contribution of urate, ammonia, soluble protein, and urea to total nitrogen excretion for experimental days 1 and 3

	Day	Urate*	Ammonia*	Soluble proteins*	Urea*	Water intake (ml day <sup>-1</sup> )
Bulbul	1	32.9±5.8	56.8±5.2	4.9±0.9	5.8±0.7	64.2±5.04
	3	48.8±3.2	37.8±3.1	8.8±1.3	4.6±0.8	38.9±3.38
Paired <i>t</i> -test <sup>†</sup>		2.72	3.37	3.79	0.8	7.8
<i>P</i>		0.02	0.008	0.004	0.43	0.0001
Grackle	1	66.5±2.8	25.8±2.5	4.4±0.6	3.3±0.3	86.1±6.7
	3	60.1±5.0	35.3±4.3	3.2±0.8	1.3±0.3	68.2±4.3
Paired <i>t</i> -test <sup>†</sup>		1.07	1.77	1.49	4.49	2.57
<i>P</i>		0.32	0.11	0.17	0.002	0.03

Values are means ± s.e.m. (N=10). Note that in both species water intake decreased significantly from day 1 to day 3.

\*% contribution to total nitrogen excretion.

<sup>†</sup>d.f.=9 for bulbuls and 8 for grackles.

However, the concentration of ammonia and urea did not differ between ureteral urine and excreta. In grackles, the concentrations of urea, soluble protein and ammonia were significantly higher in ureteral urine than in excreta, but the concentration of uric acid did not differ between ureteral urine and excreta (Table 5).

#### Plasma uric acid

The plasma concentration of uric acid was positively correlated with protein intake in grackles ( $r^2=0.65$ ,  $F_{1,6}=11.22$ ,  $P=0.015$ ; Fig. 5). However, protein intake did not affect the plasma uric acid concentration in the bulbuls ( $F_{1,6}=0.07$ ,  $P=0.8$ ; Fig. 5). The average plasma concentration of uric acid was lower in grackles ( $2.1\pm 0.3$  mg dl<sup>-1</sup>) than in bulbuls ( $3.3\pm 0.7$  mg dl<sup>-1</sup>). Thus, per unit of protein intake standardized by  $m_b^{0.75}$ , bulbuls had a higher plasma uric acid concentration than grackles (ANCOVA<sub>species</sub>:  $F_{1,12}=7.86$ ,  $P<0.02$ ).

#### Discussion

Both species had lower nitrogen requirements than expected from their body mass (Table 2). Curiously, yellow-vented bulbuls and Tristram's grackles differed significantly in the composition of the nitrogenous waste that they produced. The grackles exhibited the putative typical avian pattern. They were

uricotelic and the chemical composition of their nitrogenous waste products was relatively independent of water and nitrogen intake. The most significant aspect of our study was the demonstration of a high percentage of nitrogen excreted as ammonia in the yellow-vented bulbul. Bulbuls appeared to switch from uricotelic to ammonotelic when they ingested large amounts of water and when their protein intake was low. Furthermore, ammonotelic in bulbuls was the result of post-renal urine modification, as the concentration of urate was always higher in their ureteral urine than in their excreta (Table 4). Using the definition of Roxburgh and Pinshow (2002), bulbuls were 'apparently ammonotelic'. Here we consider the potential mechanisms that underlie the low nitrogen requirements of yellow-vented bulbuls and Tristram's grackles, we attempt to clarify the terms 'facultative ammonotelic' and 'apparent ammonotelic', and we discuss why bulbuls may be exceptional in their degree of ammonotelic.

#### Low nitrogen requirements in yellow-vented bulbuls and Tristram's grackles

Available evidence indicates that nectarivorous and frugivorous bird species have unusually low nitrogen requirements and our results were consistent with this observation (Brice and Grau, 1990; Witmer, 1998; van Tets and Nicolson, 2000; Pryor et al., 2001; Roxburgh and Pinshow,

2000; McWhorter et al., 2003). The nitrogen requirements of both bulbuls and grackles were extremely low. Because we did not recover all the nitrogen in excreta, the nitrogen requirements reported here are underestimated. However, in other experiments in which birds have been fed nectar-like diets, we have found only traces of other nitrogen-containing compounds such as creatine, creatinine, free amino acids and bile salts in excreta (McWhorter et al., 2003). We can conclude that our method underestimates nitrogen requirements only slightly (Tsahar et al., 2005).

How birds can have such low nitrogen requirements remains enigmatic. They may have lower rates of endogenous protein turnover and/or high nitrogen recycling capacity (Witmer, 1998; Pryor et al., 2001). Birds excrete urate in their urine in the form of small spherical concretions (Goldstein and Skadhauge, 2000). These concretions contain protein and inorganic ions in addition to urate (Casotti and Braun, 1997). We found that protein concentration was lower in excreta than in ureteral urine (Table 5). We hypothesize that some of the protein associated with urate spheres is digested in the lower intestine and recovered. This hypothesis is consistent with the

findings of high activities of membrane-bound digestive peptidases in the distal small intestine of the cedar waxwing *Bombycilla cedrorum* (Witmer and Martínez del Río, 2001). As protein represents only a small fraction of all nitrogen excreted, this mechanism probably contributes to the low nitrogen requirements of frugivores, but it does not explain it fully.

*Are yellow-vented bulbuls facultatively ammonotelic, apparently ammonotelic, or both?*

Preest and Beuchat (1997) were the first to challenge the long-held notion of avian uricotelic. They showed that under certain conditions Anna's hummingbirds could excrete more nitrogen as ammonia than as uric acid. They termed the ability to switch from uric acid to ammonia as the primary waste product 'facultative ammonotelic'. Roxburgh and Pinshow (2002) found that the Palestine sunbirds also switched from uric acid to ammonia excretion under some conditions. In sunbirds, ammonotelic was correlated with low protein intake, whereas in hummingbirds it was associated with low ambient temperature. Roxburgh and Pinshow (2002) found that ammonia excretion remained relatively constant, whereas uric acid excretion increased with increased protein intake. They claimed that the ammonotelic observed in sunbirds was only 'apparent'. Since uric acid was the major nitrogen waste form in the ureteral urine, no modification took place in the metabolic pathway of nitrogen waste production in the birds; hence the birds were not 'truly' ammonotelic. In sunbirds, as in bulbuls, the concentration of urate was higher in ureteral urine than in excreta. Hence, the apparent ammonotelic observed by Roxburgh and Pinshow (2002) and found in this study in bulbuls is probably the result of post-renal urine modification. The term 'apparent ammonotelic' complements the hypothesis of 'facultative ammonotelic' by offering it a potential mechanism: post-renal urine modification.

The excretion of ammonia in the yellow-vented bulbul was positively correlated with water intake. These results can explain the difference between the results of the present study and those of van Tets et al. (2001), who also studied the effect of nitrogen intake on the composition of nitrogenous waste products in the ureteral urine of yellow-vented bulbuls. The concentrations of urate and urea that they measured in the ureteral urine were similar to those we found, but those of ammonia were 4–30% lower. This discrepancy can be explained by differences in the sugar concentration of the food that the birds ingested. van Tets et al. (2001) fed their birds on a diet that contained 20% sugar, whereas we fed most of our birds a

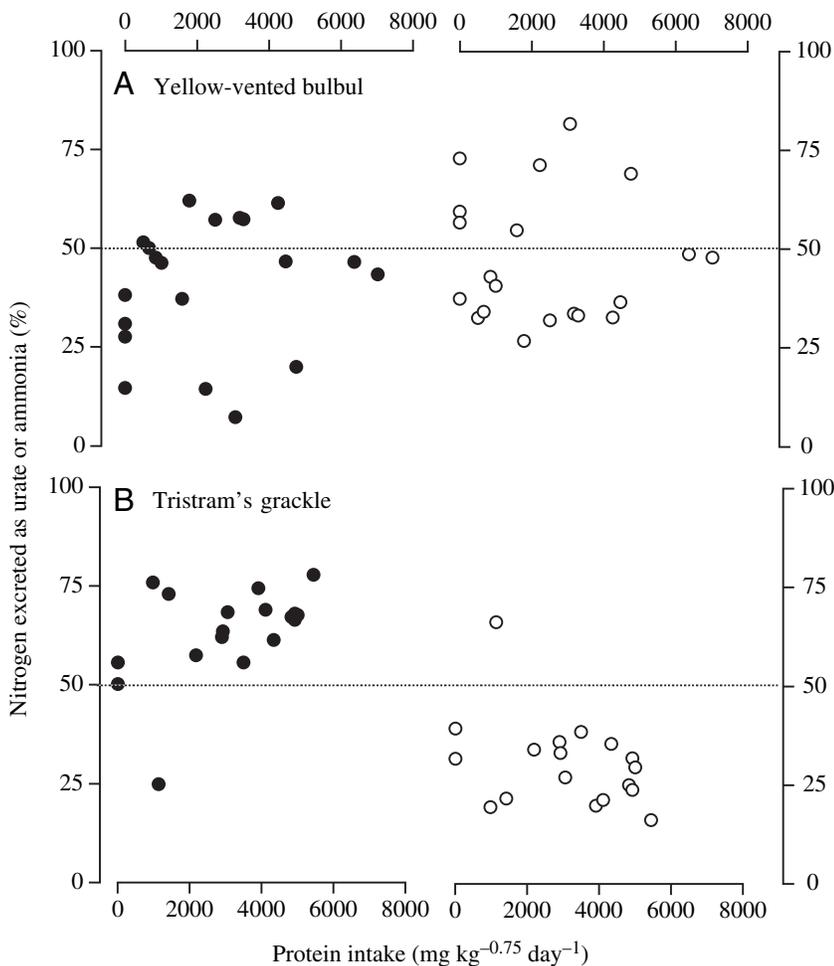


Fig. 4. Chemical composition of nitrogen excreted. Yellow-vented bulbuls were ammonotelic in roughly half of our measurements, whereas all but one of Tristram's grackles were uricotelic. Open circles, ammonia; closed circles, urate.

Table 5. Comparison between the concentrations of nitrogenous waste products in ureteral urine and in excreta, of yellow-vented bulbuls and Tristram's grackles

	Concentration of waste product (mg ml <sup>-1</sup> )			
	Urate*	Ammonia*	Soluble proteins*	Urea*
<b>Bulbul</b>				
Excreta	0.44±0.07	0.17±0.02	0.12±0.02	0.03±0.01
Ureteral urine	1.14±0.1	0.36±0.1	0.32±0.06	0.15±0.06
Paired <i>t</i>	7.8	2.0	2.7	2.2
d.f.	13	13	10	3
<i>P</i>	>0.0001	0.07	0.02	0.1
<b>Grackle</b>				
Excreta	1.32±0.17	0.22±0.03	0.14±0.02	0.03±0.01
Ureteral urine	1.27±0.14	0.36±0.05	0.63±0.13	0.12±0.03
Paired <i>t</i>	0.2	3.32	3.8	3.3
d.f.	16	17	16	16
<i>P</i>	0.6	0.004	0.002	0.004

\*Values are means ± S.E.M.

The concentration of urate and ammonia can be transformed to the concentration of nitrogen excreted as urate and ammonia (in mg N ml<sup>-1</sup>) by multiplying the numbers in this table by 0.334 and 0.823, respectively.

diet that contained only 10% sugar. The water intake of birds on a 20% sugar diet is 50% lower than that of birds fed on a 10% sugar diet (Fig. 1). Although such a correlation is expected from the observation that ammonia is highly toxic, and its disposal requires large amounts of water, the mechanisms that lead to it are unclear.

The percentage of nitrogen excreted as urate and ammonia was not affected by water intake in Tristram's grackles.

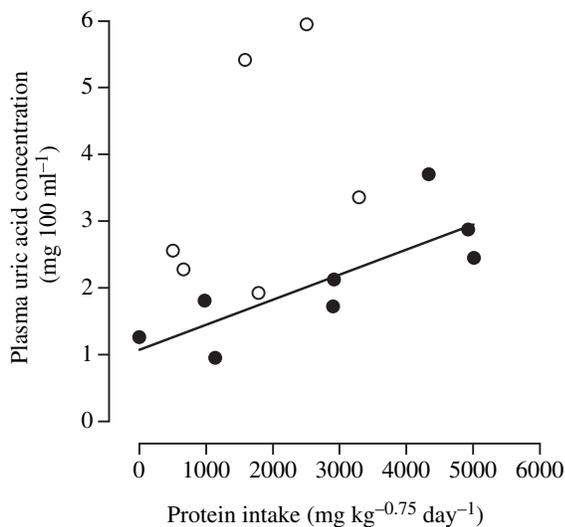


Fig. 5. The effect of protein intake on plasma uric acid. For a given protein intake, the plasma concentration of uric acid was significantly higher in yellow-vented bulbuls (open circles) than in Tristram's grackles (closed circles). Plasma uric acid concentration was positively correlated with protein intake in Tristram's grackles, ( $y=0.0004x+1.07$ ,  $F_{1,6}=11.22$ ,  $r^2=0.65$ ,  $P=0.015$ ), but not in yellow-vented bulbuls ( $F_{1,5}=0.07$ ,  $P=0.8$ ). We standardized protein intake by mass<sup>0.75</sup> to place both species along an axis of similar magnitude.

However, we only tested this species with 20% sugar. Hence, grackles had relatively lower and less variable water intakes than bulbuls. It is possible that with a higher water intake the grackles would also have demonstrated ammonotelicity and that with a larger range of intakes, ammonotelicity and water intake would have been positively correlated.

#### *How do bulbuls and sunbirds reduce the urate content in excreta: the mechanisms behind apparent ammonotelicity*

The modification of urine in the lower gut of Palestine sunbirds and yellow-vented bulbuls may be the result of two non-exclusive mechanisms: bacterial breakdown of uric acid or reabsorption of uric acid by the birds. We currently have no strong evidence for or against either of these mechanisms. Hence this section must be conceived more as a description of hypotheses in need of testing than an account of established mechanisms. Preest et al. (2003) documented microbial degradation of potassium urate (but curiously not of sodium urate) by microbes extracted from the distal intestine of Anna's hummingbirds. Bulbuls also have microbes with uricolytic activity in their lower gastrointestinal tract (E. Tsahar, unpublished data). Microbial activity in the lower gut can have two effects: (1) it can lower the concentration of urate, and (2) it can increase the concentration of ammonia, which is a by-product of the microbial degradation of urate (Karasawa et al., 1988). If birds recover the ammonia resulting from microbial urate degradation, then this mechanism can improve the nitrogen economy of birds. Some bird species have large ceca that contain sizeable populations of microorganisms (Clench, 1999). In these species, nitrogen conservation through the breakdown of uric acid is of significant physiological importance (Mortensen and Tindel, 1981; Campbell and Braun, 1986; Karasawa et al., 1988; Karasawa et al., 1993; Son and Karasawa, 2000). Bulbuls and sunbirds have tiny vestigial

ceca and hummingbirds have no cecum at all. The physiological importance of the microbial degradation of urate, if any, in these species remains unknown.

An alternative mechanism that can reduce the concentration of uric acid in excreta is the potential presence of a specific urate transporter in the lower guts of birds. Although we lack the evidence, we speculate that the bulbuls' lower intestine might have the capacity to reabsorb urate. This speculation prompts the question, why would it be advantageous for birds to recover a nitrogenous metabolic waste? Although uric acid is considered primarily as a nitrogenous waste, it also has a major function as a powerful antioxidant in both mammals (Davies et al., 1986; Becker, 1993; Schlotte et al., 1998; Spitsin et al., 2000) and birds (Simoyi et al., 2002; Somoyi et al., 2003). Uric acid may be an especially important antioxidant in mammalian species, such as humans, that do not express the enzyme L-gulonolactone oxidase (GLO) and hence lack the ability to synthesize ascorbic acid (Chatterjee, 1973), another potent antioxidant. Although GLO expression has not been measured in yellow-vented bulbuls, these birds are members of a bird lineage that lacks it. Tristram's grackles belong to a lineage that expresses the enzyme (Martínez del Rio, 1997). We suggest that uric acid may shift its functional role from a waste product to a useful antioxidant in birds species that lack the ability to synthesize ascorbic acid.

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